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J. FRANK DANIEL
1873-1942

CONTENTS

DEDICATION

I⊤ IS FITTING that this volume of the University of California Publications in Zoölogy should be dedicated to the late Professor J. Frank Daniel, who was, at the time of his death, chairman of both the Editorial Board of this series and the Department of Zoölogy, and who had upheld the highest standards in scholarly and scientific work during his academic career of more than thirty years at the University of California. The papers included in the volume have been written by students of Dr. Daniel and many of the investigations here reported were conducted in his laboratory of experimental morphogenesis, a field in which Daniel developed undergraduate and graduate instruction at Berkeley and one in which he made a number of studies.

Daniel and his group of students, including F. G. Gilchrist, A. L. Alderman, and others in addition to those represented here, have investigated problems primarily in the development of two widely distributed amphibian forms: the Pacific Coast newt, *Triturus torosus*, and the Pacific tree frog, *Hyla regilla*. The problems included localization of materials, establishment of pattern, formative movements, gradients, determination, regulation, and the role of the endocrines in morphogenesis. They have dealt with a considerable period of amphibian development—from the ovarian egg to the larva—and with the developmental mechanics of a large number of systems such as the eye, limb, thyroid, hypophysis, musculature, and nervous system. Daniel himself was especially interested in the causal factors in the establishment of pattern in the unfertilized amphibian ovum and later in the zygote, and in the relationship of the basic ovarian and zygotic patterns to those in the future embryo.

On behalf of the students and colleagues of Professor Daniel we affectionately dedicate this volume to his memory.

S. J. HOLMES
RICHARD M. EAKIN

THE MECHANISM OF AMPHIBIAN GASTRULATION

I. GASTRULATION-PROMOTING INTERACTIONS BETWEEN VARIOUS REGIONS OF AN AN JRAN EGG (HYLA REGILLA)

BY

A. MANDEL SCHECHTMAN

CONTENTS

THE MECHANISM OF AMPHIBIAN GASTRULATION

I. Gastrulation-Promoting Interactions between Various Regions of an Anuran Egg (Hyla Regilla)

BY

A. MANDEL SCHECHTMAN

(Contribution from the Department of Zoölogy, University of California, Los Angeles)

INTRODUCTION

OUR KNOWLEDGE of the structural and kinematic aspects of gastrulation in the Amphibia reached a high degree of completeness in the classic paper of Vogt (1929). The causal-analytical aspects are far more complex and still are controversial, although much information has been accumulated since Vogt (1922) attempted to study causal factors in gastrulation by surgical methods. General discussions of recent work have been published by Spemann (1938), Pasteels (1940), and Waddington (1940). The dominant theory of the nature of the gastrulation movements is that of strict autonomy, which was advocated by Roux (1895), and which has recently been characterized by Spemann (1938) as follows:

However harmonious the process of motion may be by which the material for the chief organs arrives at the final place, and however accurately the single movements may fit together—for indeed they must fit together if the final result is to be achieved—they are, nevertheless, no longer causally connected, at least from the beginning of gastrulation onward. Rather, each part has already previously had impressed upon it in some way or other direction and limitation of movement. The movements are regulated, not in a coarse mechanical manner, through pressure and pull of the simple parts, but they are ordered according to a definite plan (in the sense of von Uexküll). After an exact patterned arrangement, they take their course according to independent formative tendencies which originate in the parts themselves. Thus we find in the gastrula stage a mosaic, a pattern of parts with definite formative tendencies from which the formative tendencies of the whole must necessarily follow. (Pp. 106–107.)

There are a number of observations, however, which do not clearly lend themselves to such a view; in fact, they suggest the possibility that gastrulation involves more than an intricately arranged mosaic each element of which acts at the right time and in the right way. These observations are considered in some detail below (pp. 25–28); at this place we need only mention a typical instance. During normal gastrulation the presumptive chordal material (centered in the dorsal blastoporal lip) turns into the interior of the egg and elongates into the familiar rodlike chorda. But the dorsal lip, after it has been transplanted into the presumptive ectodermal region, does not carry out its customary involution and invagination (Mangold, 1923; Lehmann, 1932; Töndury, 1936; and others). It does elongate, but *away* from the surface of the egg, rather than under the surface, forming a fingerlike appendage. When,

however, the dorsal lip is transplanted into the ventral blastoporal lip it "invaginates with the greatest of ease" (Spemann, 1938, p. 151). This situation has been interpreted in two ways. The more common interpretation is that the presumptive chorda of the dorsal lip has an inherent capacity for involution and invagination which is not manifested when the dorsal lip is in the presumptive ectoderm, because of mechanical hindrances such as the resistance offered by the underlying invaginating mesoderm and entoderm of the host. The second interpretation is that the presumptive chorda does not invaginate because it has little or no inherent capacity for such movements; it requires some kind of aid which the presumptive ectoderm cannot supply but which in normal gastrulation is supplied by another region of the egg. The central aim of the present study is to ascertain whether such auxiliary relationships exist.

In referring to the movements of gastrulation we are constantly liable to the danger of misunderstanding, for the terms involved are frequently used in dissimilar senses. Thus Weiss (1939) uses the term "invagination" to designate the movements of the marginal zone, and "sinking-in" to designate those of the vegetative material (p. 417), whereas to Vogt (1929) invagination includes two main movements: (1) in-sinking (*Einstülpung*) of the egg surface, and (2) forward migration (*Vordringen*); in other words, a displacement of any material from a superficial position into the interior of the egg as well as the displacement of material originally internal to a more anterior position. In the Anura a comparatively large proportion of the presumptive mesoderm is situated internally at the onset of gastrulation. This material gradually progresses toward the animal pole, moving along the inner surface of the animal hemisphere. This is obviously a broad use of the term "invagination." But it is not the same as "gastrulation," for the latter process includes additional movements such as extension, dorsalward convergence, and constriction.

It is important to keep in mind that the term "invagination" and, indeed, all the other terms used are descriptive of kinematics. They do not indicate the nature or location of the forces involved in the various movements. Thus it is permissible and customary to say that the presumptive pharyngeal entoderm invaginates to a new position close to the animal pole. This is merely stating that the material passes inward (kinematics); it does not indicate whether it moves itself or is pulled or pushed in by neighboring materials (kinetics). With respect to the presumptive pharyngeal entoderm, the invagination process is clearly complex in its causal-analytical aspects. It involves several dissimilar mechanisms. The dorsal blastoporal groove begins in the presumptive entoderm as a depression of the egg surface and is associated with the presence of flask- or club-shaped cells around the apex of the groove (Goodale, 1911). It seems apparent from the work of Vogt (1922a) and others that the groove is formed, at least in part, by the active contraction of the outer ends of these cells. But the further invagination of the presumptive pharyngeal entoderm involves different mechanisms; for, as the groove deepens, the original flask-shaped cells disappear and no new ones form which can account

for the continued invagination (see figs. 30, 34, and 35, Vogt, 1929; and plate 58, figs. 29, 30, and 31, Daniel and Yarwood, 1939). In later phases of gastrulation the presumptive pharyngeal cells tend to flatten and spread (Goodale, 1911; Vogt, 1929; Lehmann, 1932). It seems probable from the data presented in the present paper that the extension of the presumptive chorda material is instrumental in carrying the presumptive pharyngeal entoderm to its anterior position.

Extension (extension, elongation, Goodale; *Streckung, Staffelung,* Vogt). As the dorsal blastoporal groove becomes evident, or slightly before this, the superficial region of the marginal zone (*Randzone,* Vogt) begins to move toward the groove. The term "marginal zone" indicates the presumptive chorda-mesoderm-entoderm complex at the junction of the roof and floor of the blastula or early gastrula. The extension sets in first in the dorsal region of the marginal zone and then appears progessively in more and more ventral regions. This is a simple expression in comparison with "invagination"; it refers to the elongation of a mass of cells in one direction in the same sense as when we refer to the stretching of a rubber band. However, it does not imply that any external tension is necessarily involved, nor that the extended material need simultaneously become narrower as in the rubber band. The stretching marginal zone does become narrower as it approaches the blastoporal lip, but after it has turned into the egg it may *spread* as it stretches (as in the presumptive lateral plate mesoderm) or it may continue to stretch and become narrower (presumptive chorda). All experimental work indicates that the extension of the marginal zone is a self-stretching which results from forces inherent in the material itself.

Involution (embolic invagination, Jordan; *Einrollung, Umschlag,* Vogt) refers to the rotation of a material upon itself so that its movement is directed toward the interior of the egg. This was demonstrated by Smith (1914) by the use of vital-stain marks. It is not the same as "invagination," although the two terms are sometimes used as synonyms. As the marginal zone extends toward the blastoporal groove, the portions nearest the groove turn upon themselves and then invaginate into the interior. This is followed by successive portions of the marginal zone, so that the blastoporal lips are continually changing their material constitution during gastrulation. The term "involution" is sometimes used to indicate the turning inward *and* subsequent invagination, but, as has already been pointed out, certain portions of the gastrula invaginate but do not perform any involution movements.

Dorsalward convergence (confluence, Smith; *dorsal Raffung,* Vogt). Smith's vital-stain experiments showed that material of the marginal zone moves toward the dorsal midline as it involutes and invaginates. This is most clearly discernible in laterally located portions of the presumptive chorda and somites, and to a lesser degree in the presumptive lateral plate mesoderm, but all portions of the marginal zone move dorsalward to some degree during gastrulation. This automatically produces *ventral divergence* (Vogt), for any material which converges dorsalward is simultaneously diverging from the mid-ventral line.
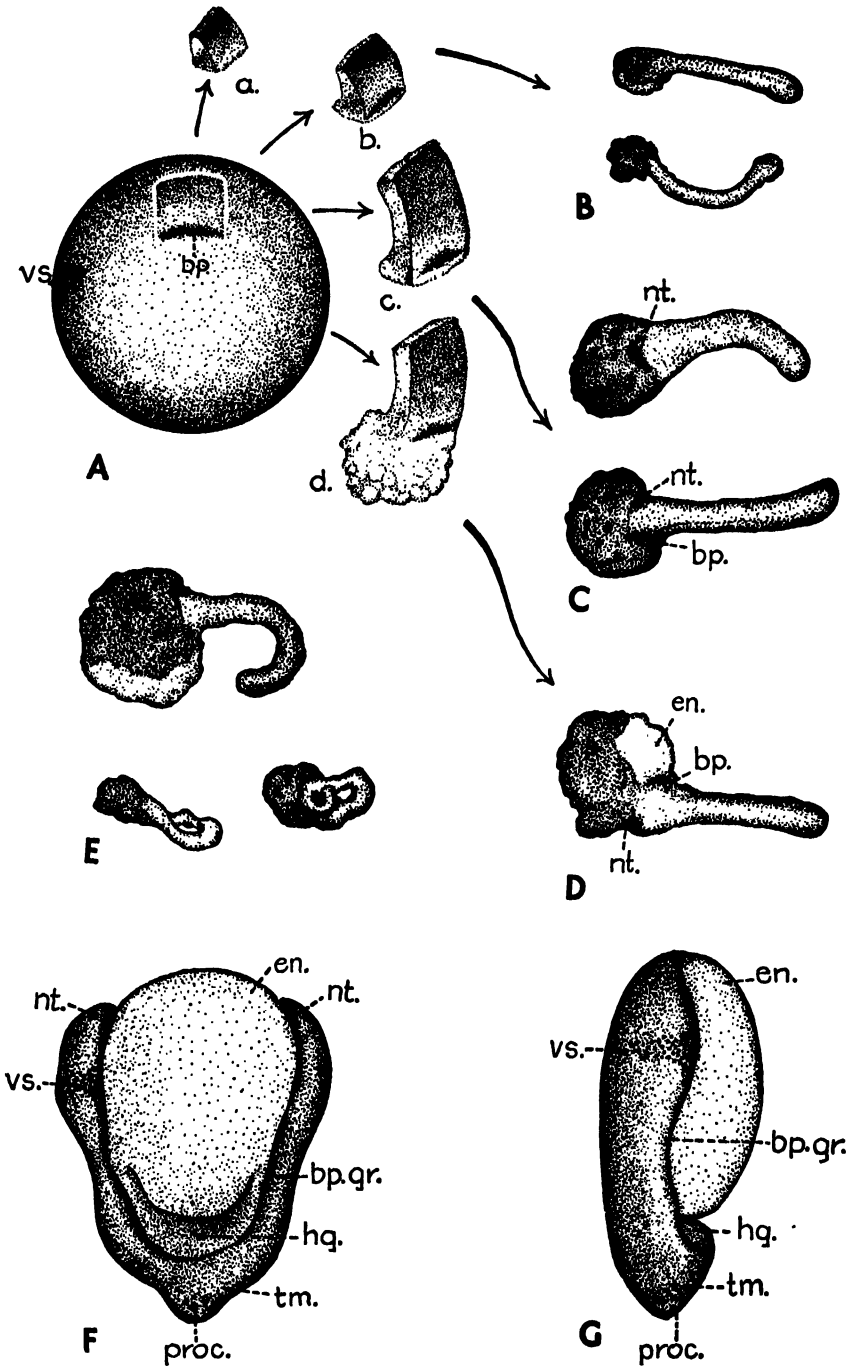
FIG. 1

(For explanation of figure 1 see foot of following page.)

Constriction (convergence, Jordan; *konzentrisches Urmundschluss*, Vogt). Goodale (1911), Smith (1914), and Vogt (1929) showed that the gradual closure of the blastopore is accomplished by the constriction of the blastoporal lips as they stretch over the yolk mass toward the vegetative pole. Vogt (1922*a*) was of the opinion that constriction is a mechanical consequence of the extension (stretching) and simultaneous narrowing of every portion of the marginal zone. The present experiments indicate that an additional factor—a pull or tension exerted by the dorsal blastoporal lip—plays an important part in constriction.

Epiboly. In the present paper we follow Vogt's usage of this term to indicate the increase in areal extent of the presumptive ectoderm during gastrulation. The term is sometimes used to designate the progressive envelopment of the yolk mass by the blastoporal lips (constriction, in the present usage).

MATERIALS AND GENERAL METHOD

The eggs of *Hyla regilla* (Baird and Girard) were used throughout. The operations were performed on groups of 8 to 12 eggs, with a total of 30 to 70 eggs for each experiment, except where smaller numbers are indicated in the subsequent account. The jelly and egg membrane (chorion, Daniel and Yarwood, 1939; *Dotterhäutchen* of the German authors) were removed in the late blastula or early gastrula stages with the aid of fine-pointed forceps. After washing in 4 to 6 changes of sterile Holtfreter's solution (Holtfreter, 1931), the eggs were stored for 1 to 2 hours in a small icebox placed on the operating table; from this icebox a few eggs were removed as required for each operation, so that the stage of development was kept as constant as possible. All operations were carried out in sterile Holtfreter's solution with the aid of black-glass needles. It was found advantageous to work upon a substrate of beeswax darkened with lampblack, but most of the eggs were then transferred to a 3-per cent agar substrate. At room temperature (18° to 25° C.) the eggs required 12 to 16 hours to pass from the earliest gastrula to the middle neurula stage (sole-shaped neural plate). Disintegration rarely occurred during this short period; indeed, many cultures were maintained for 4 to 7 days longer in good condition. The details of individual operations are given in the account which follows. All figures were drawn with the aid of a camera lucida.

Fig. 1. A. Various types of explants involving the dorsal lip. *a.*, dorsal lip without circumblastoporal material; *b.*, dorsal lip with circumblastoporal material; *c.*, dorsal lip with circumblastoporal material and more presumptive ectoderm; *d.*, dorsal lip with more presumptive ectoderm and entoderm.

    B. C. D. The explants after 14 to 16 hours of culture.

    E. The explants after 18 to 24 hours of culture.

    F. G. Ring embryo produced by removal of the dorsal lip, as seen from above (F) and from the side (G). *bp.*, blastopore; *bp. gr.*, blastoporal groove; *en.*, entoderm; *hg.*, hindgut entoderm; *nt.*, neural tissue; *proc.*, proctodeum; *tm.*, tail mesoderm; *vs.*, vital-stain mark shown in figure 1, A, F, G.
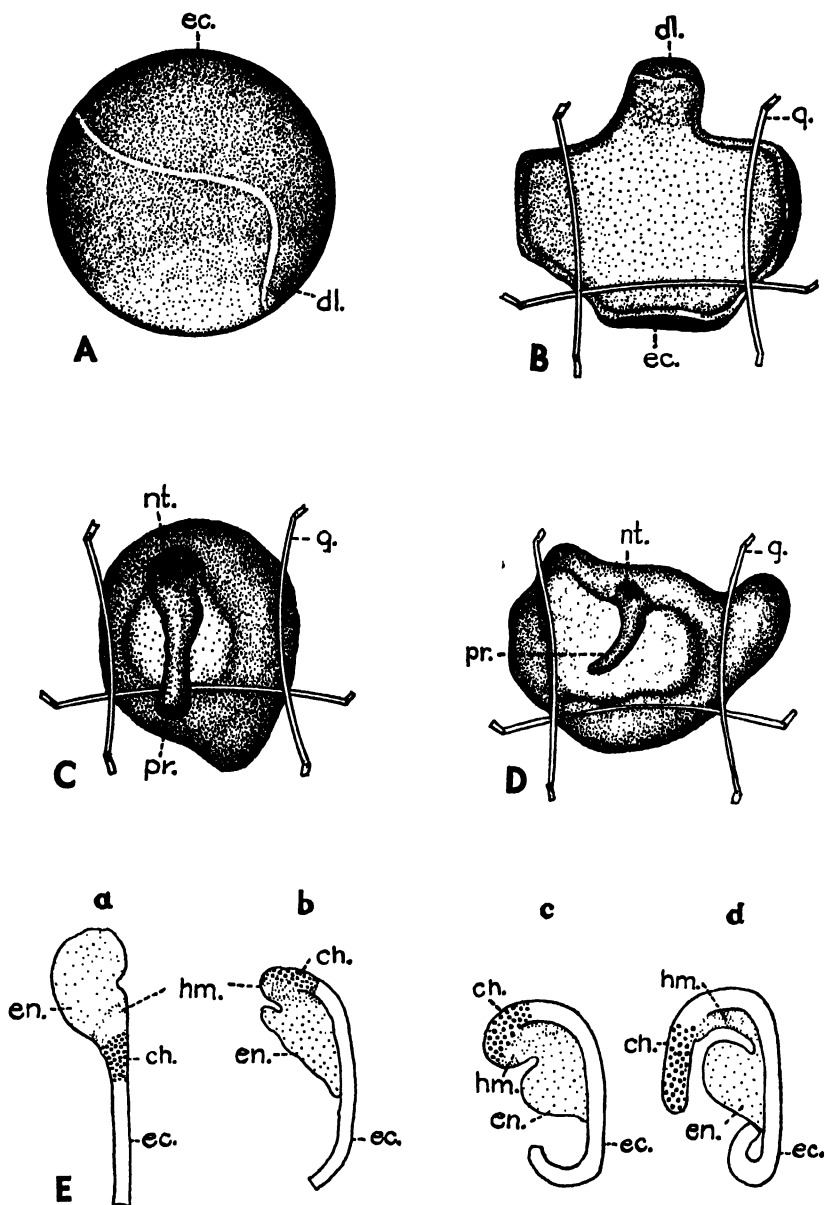
Fig. 2. A. Incision made in order to explant the dorsal lip together with a large amount of presumptive ectoderm.

B. The explant fastened to wax substrate.

C. D. Explants after 16 hours of culture. The explant shown in C included a larger amount of entoderm than the one shown in D.

E. Diagrams showing successive stages (*a–d*) in the development o⁵ the explants shown in B, C, D. *ch.*, presumptive chorda (coarse stippling); *dl.*, dorsal lip; *ec.*, presumptive ectoderm; *en.*, presumptive entoderm (sparsely stippled); *g.*, tinfoil girder; *hm.*, presumptive head mesoderm (fine stippling); *nt.*, neural tissue; *pr.*, presumptive chordal projection.

BEHAVIOR OF THE DORSAL AND LATERAL PORTIONS OF THE MARGINAL ZONE UNDER VARIOUS EXPERIMENTAL CONDITIONS

Experiment I: The explanted dorsal lip.—The dorsal lip was explanted from very early gastrulae in which the blastoporal groove (*bp.*, fig. 1, A) was a short, shallow crescent. Four types of operation were made, involving increases in the amount of presumptive ectoderm and entoderm included with the dorsal lip explants (fig. 1, A, *a–d*). In the first type of explant (*a*) the ventralmost cut was made just *above* the blastoporal groove; in the second (*b*), the cut passed just *below* the groove: in the third (*c*), more of presumptive ectoderm was included; whereas the fourth (*d*) included a good deal of both presumptive ectoderm and entoderm. All the explants at first rounded up, but 5 to 6 hours later the outer, pigmented surface began to bulge outward as a blunt hillock. This extension continued until the clublike projections shown in figure 1, B, C, and D, were formed. Some of the projections were curved; others were straight. The specimens shown are the longest that were obtained; many reached only two-thirds this length. Even the longest and straightest specimens eventually underwent curling and formed irregular masses (fig. 1, E). This curling is not the equivalent of the normal involution of the dorsal lip, for in various specimens it is extremely irregular. It may be absent during the first 12 hours of culture, and most of the curling takes place *after* the elongation rather than with it.

In all explants containing the circumblastoporal material (fig. 1, A, *b–d*) the blastoporal groove remained visible and actually deepened for the first 3 to 6 hours of culture. It was subsequently obliterated in most specimens, although a clear pit remained visible in the larger explants even after 16 hours (*bp.*, fig. 1, C and D). Small patches of neural tissue, clearly visible in the larger explants (*nt.*, fig. 1, C and D), were verified in sectioned material.

The donor eggs, lacking the dorsal lip, develop in a constant manner (fig. 1, F and G). In the absence of the dorsal lip the lateral and ventral parts of the blastoporal groove develop approximately in unison with those of normal eggs. A vital-stain mark (*vs.*, fig. 1, A) placed on the lateral marginal zone stretched toward the groove and turned inward (*vs.*, fig. 1, F); the portion extending into the groove was strongly stretched in the dorsoventral direction of the egg. Now, in the normal gastrula a mark placed in this same location also stretches toward and around the blastoporal lip, but in addition is carried dorsalward and finally appears in the embryo as an elongate streak extending in an anterior-posterior direction. Hence in the present ring embryos the lateral and ventral marginal zones are capable of involution and stretching but do not converge dorsalward nor constrict over the yolk mass.

The greater part of the entodermal mass remains exposed to view even after days of culture. The presumptive hind-gut mesoderm (*hg.*, fig. 1, F and G) is of a grayish color and set off from the main yolk mass by a deep groove. In some specimens this groove is obliterated by the fusion of its anterior and posterior walls (*hg.*, pl. 1, fig. 3). No neural plate was observed in any of
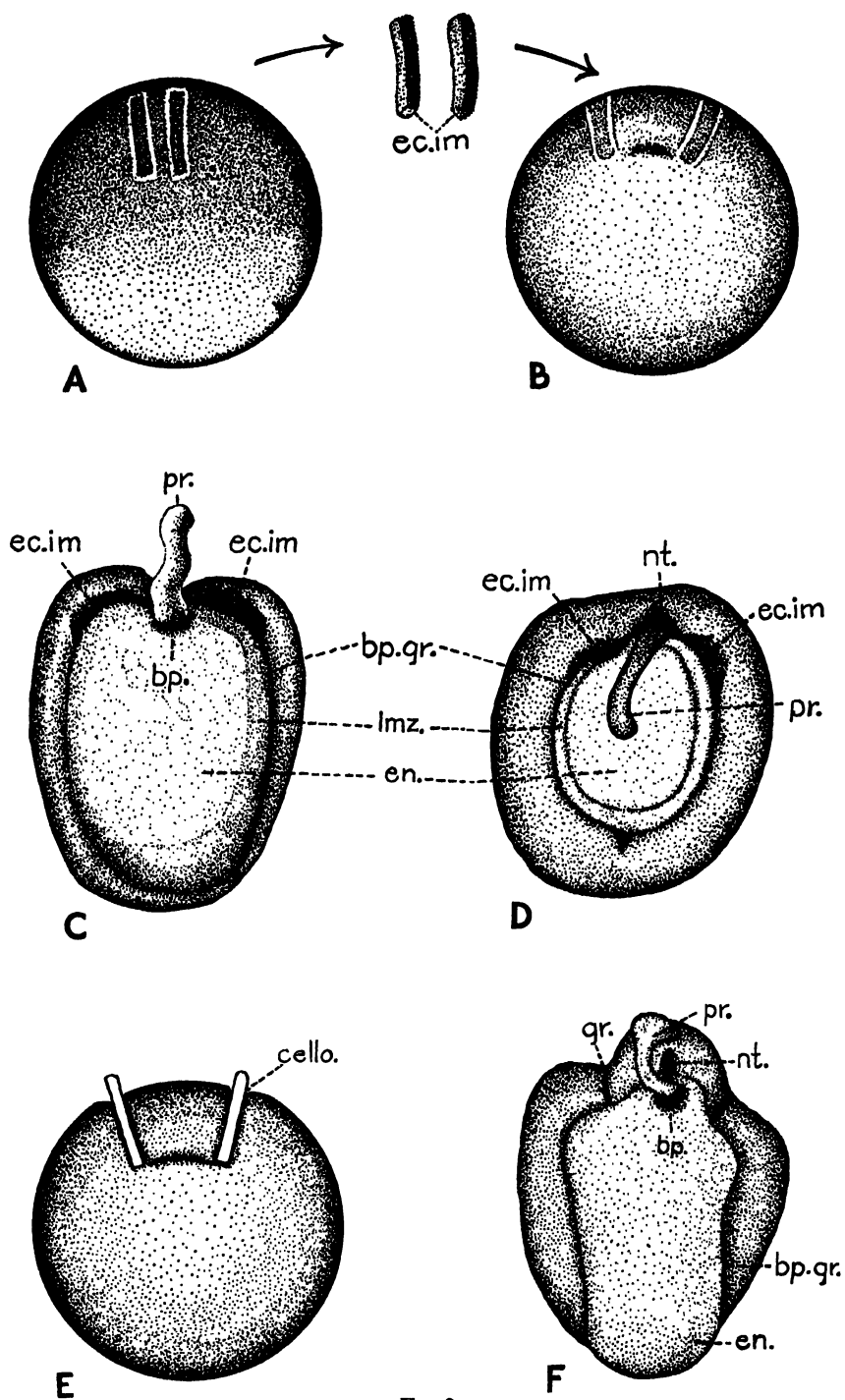
FIG. 3

(For explanation of figure 3 see foot of following page.)

these specimens, although elongate patches of tissue which apparently were neural were present bilaterally at the anterior ends (*nt.*, fig. 1, F). These embryos are essentially like those described by Dalcq (1940). Their internal structure as seen in sections is so like that of other ring embryos described in experiment III that the same figures may be used to illustrate both cases (pl. 1, figs. 2, 3). The internal parts of the marginal zone spread into the blasto-coele so that a layer of mesoderm (*m.*) lies between the presumptive entoderm and ectoderm. In later development, somites appeared in the two rounded ridges bordering the yolk mass, and the posterior part of the marginal zone (*tm.*, fig. 1, F and G) developed into an elongate tail with a ventral fin-fold. The presumptive tail mesoderm has thus retained its primitive position; it has not moved dorsalward as it does in the normal embryo.

Experiment II: The dorsal lip explanted together with a large amount of presumptive ectoderm.—The explants described above rapidly rounded up into spheroidal masses. It is possible that the presumptive chordal anlage did not turn upon itself (involute) because it was impeded mechanically by the curling presumptive ectoderm. Attempts were therefore made to delay the curling. The dorsal lip was excised together with a small amount of sub-blastoporal material and most of the presumptive ectoderm (fig. 2, A). The explants were laid on the outer, pigmented surface and fastened down to the wax substrate by means of tinfoil girders coated with beeswax (fig. 2, B). Care was taken that the girders pressed against but did not cut the tissue.

The girders delayed the curling of the presumptive ectoderm to various degrees in different specimens; but in all these the region of the dorsal lip elongated into a fingerlike projection (*pr.*, fig. 2, D) and curled upon itself so that it lay above the main body of the explant. This curling was not true involution, as was shown by dissections. The presumptive chorda in normal development takes up a position between presumptive ectoderm and entoderm. In the present explants it lies on a layer of entoderm which in turn lies upon the ectoderm. In order to verify this relationship a series of similar explants was prepared with the inclusion of a larger amount of sub-blastoporal ento-derm. It was thought possible that the weight of the yolk-rich cells would aid in preventing the postoperational curling of the dorsal lip. The results, how-ever, were the same as those just described: the presumptive chorda projected over the original outer surface of the yolk mass (fig. 2, C). These specimens were studied in sections as well as by dissection of the living material.

Figure 2, E shows diagrammatically several successive stages. The ento-derm first extended itself over the flattened presumptive ectoderm so that

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Fig. 3. A. B. Implantation of strips of presumptive ectoderm (*ec. im.*) into slits made through the marginal zone on both sides of the dorsal lip.

C. D. Two types of ring embryos resulting from the foregoing operation.

E. Insertion of two strips of cellophane (*cello.*) into slits made through the mar-ginal zone on both sides of the dorsal lip.

F. Ring embryo resulting from the foregoing operation. *bp.*, blastopore; *bp. gr.*, blastoporal groove; *ec. im.*, ectodermal implant; *en.*, entoderm; *gr.*, groove left by cello-phane; *lmz.*, exposed portion of lateral marginal zone; *nt.*, neural tissue; *pr.*, presumptive chordal projection.

the dorsal lip pointed upward. The circumblastoporal material invaginated to form a pocketlike cavity, while the presumptive chordal region extended over the entoderm. Plate 1, fig. 1 shows a typical sagittal section in which the abnormal relationships of the presumptive ectoderm, chorda, and entoderm are evident. A well-developed neural mass (*nt.*) lies at the base of the projection (*pr.*). In some specimens a deep blastoporal pit is present (*bp.*), but in others the walls of the pit are so close together that no actual cavity is visible.

Experiment III. The dorsal lip isolated *in situ*.—In order to provide the dorsal lip with an abundance of presumptive ectoderm under which it might pass, attempts were made to isolate the dorsal lip while it retained its normal position in the egg. Deep slits were made through the marginal zone on both sides of the dorsal lip and various materials were inserted into the slits. It was surprising to find that two narrow strips of presumptive ectoderm from the early gastrula were capable of effecting the isolation of the dorsal lip, so that it behaved as if it had been completely removed from the egg. In this operation nothing was removed from the marginal zone; the slits were cut with fine-pointed glass needles and the presumptive ectoderm was immediately inserted (fig. 3, A and B). Healing was completed within 10 to 15 minutes. The dorsal lip now retained its normal connections with the presumptive ectoderm above and the entoderm below. Observations made 5 hours after the operation showed that the outer surface of the presumptive chordal region was raised into a round hillock, which in the following 8 hours became an elongate projection (*pr.*, fig. 3, C and D). The projection may extend dorsalward, away from the egg surface, as in figure 3, C, or may lie on the outer surface of the yolk mass as in figure 3, D. In some of the eggs the distal tip may become buried in the yolk mass. At the base of the projection there is a small mass of neural tissue on the dorsal side (*nt.*, fig. 3, D) and a deep blastoporal pocket on the ventral side (*bp.*, fig. 3, C). The rest of the egg developed as when the dorsal lip was completely removed, forming a ring embryo. In most specimens there was hardly any constriction of the blastoporal lips over the yolk mass (fig. 3, C), but in 4 out of 17 there occurred a moderate degree of constriction (fig. 3, D). The implanted strips of presumptive ectoderm were stained with nile blue sulfate in 6 specimens so that their behavior could be followed. They turned in around the blastoporal lip and stretched in a dorsoventral direction (*ec. im.*, fig. 3, C and D).

A typical mid-sagittal section is shown in plate 1, figure 3. The entoderm (*en.*) rests upon a bed of mesoderm (*m.*), which is continuous posteriorly with a solid, wedge-shaped mass of tail mesoderm (*tm.*). The presumptive hind-gut entoderm in the present specimen has fused into a tonguelike body (*hg.*); in others a deep groove separates this mass into two layers. The neural tissue (*nt.*) is well differentiated, and the blastoporal pit (*bp.*) is clear although in many specimens it is almost completely occluded.

A cross section taken approximately through the middle of an embryo is shown in plate 1, figure 2. The central portion of the mesodermal layer (*m.*) is composed of loosely arranged cells, whereas the lateral portions (*so. m.*)

have a more compact appearance. In later development these lateral portions of the mesoderm developed into somites. The presumptive epidermis (*ec.*) forms a smooth layer; as is well known, the presumptive ectoderm develops into a highly wrinkled mass when *explanted* (Spemann, 1938), but when in contact with mesoderm its tendency to spread is kept under control.

An interpretation of the movements in these embryos is presented in figure 4, A–D, based on dissections of living material and sectioned specimens. The results were essentially like those described in experiments I and II. The
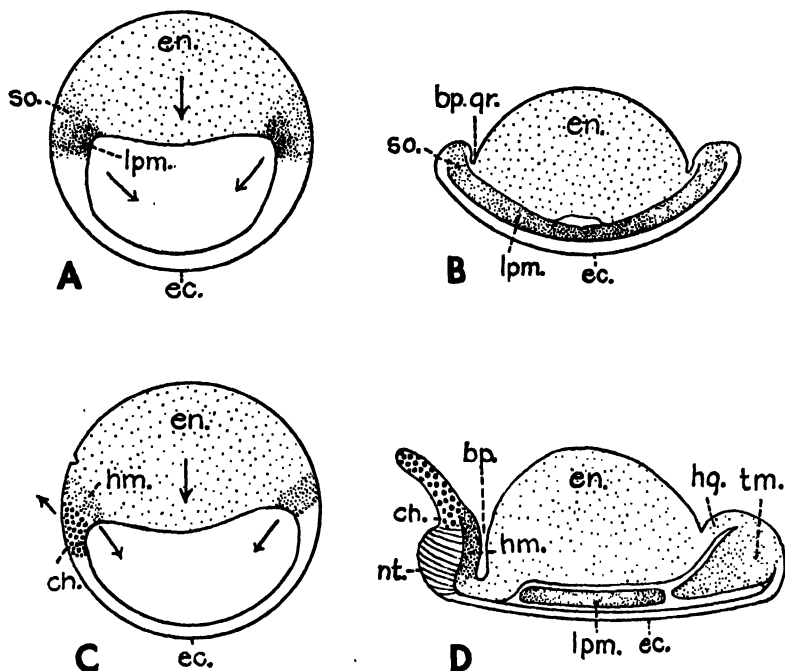
Fig. 4. Diagrams illustrating movements of the marginal zone materials in the ring embryos shown in transverse section (A, B) and in sagittal section (C, D). *bp.*, blastoporal pocket; *bp. gr.*, blastoporal groove; *ch.*, presumptive chorda (coarse stippling); *ec.*, presumptive ectoderm (unshaded); *en.*, presumptive entoderm (sparsely stippled); *hg.*, hindgut entoderm; *hm.*, presumptive head mesoderm (fine stippling); *lpm.*, lateral plate mesoderm; *nt.*, neural tissue; *so.*, somite mesoderm; *tm.*, tail mesoderm. Arrows show the directions of movements in presumptive anlagen.

presumptive chordal region extended away from the surface. The presumptive pharyngeal entoderm and head mesoderm invaginated to a limited degree. The lateral and ventral portions of the superficial marginal zone turned inward, and the internally situated portions of the marginal zone migrated into the blastocoele along the presumptive ectoderm. There was neither constriction nor dorsalward convergence to any appreciable extent.

In another series of early gastrulae thin sheets of cellophane were inserted into the slits on both sides of the dorsal lip (fig. 3, E). The cut surfaces did not adhere to the cellophane, but rounded up. Essentially the same results were obtained as with strips of presumptive ectoderm, except that the dorso-
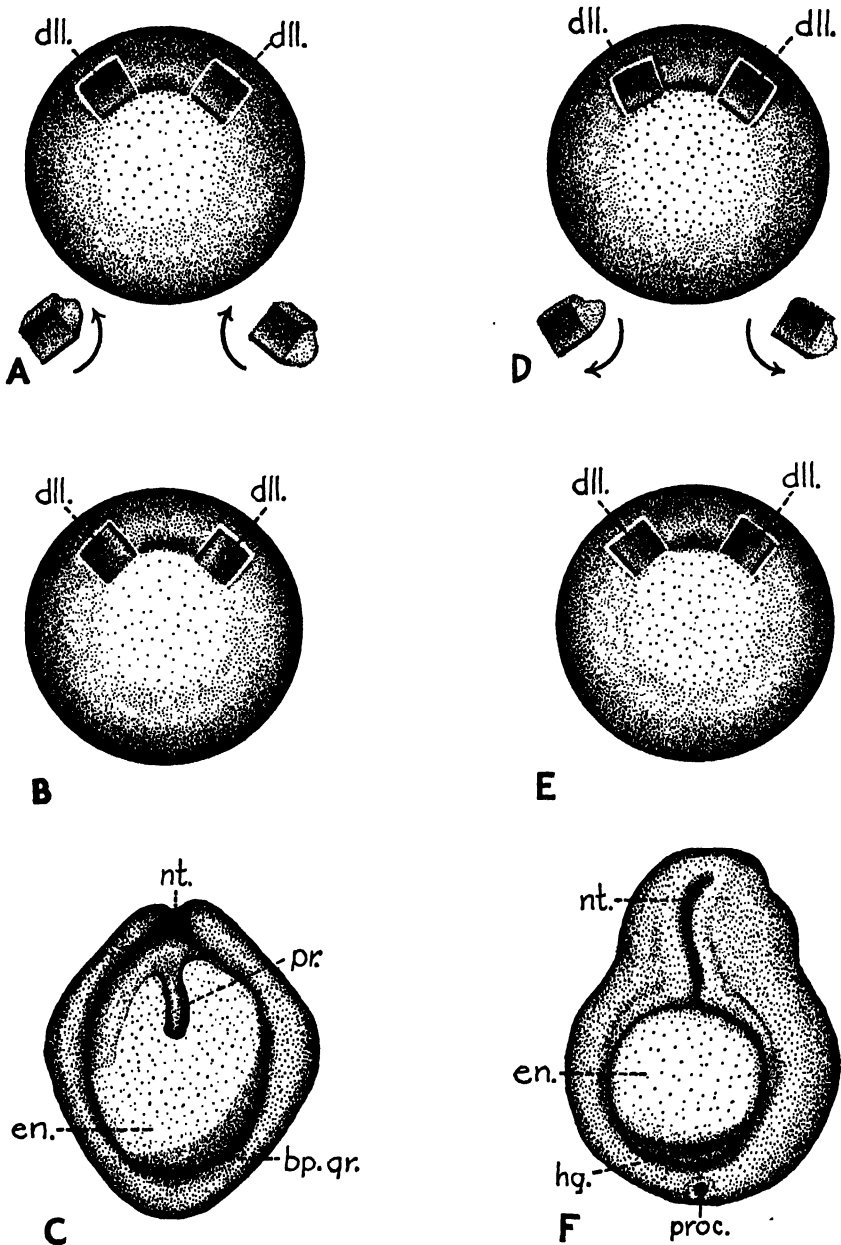
Fig. 5. A. B. Rotation of the dorsolateral marginal zones (*dll.*) 90° as shown by curved arrows. The squares are then replaced so that the original presumptive entodermal edge (shown as solid line in B) flanks the dorsal lip.

   C. Ring embryo resulting from the foregoing operation.

   D. E. Rotation of dorsolateral marginal zones (*dll.*) 90° so that the presumptive entodermal edge (solid line in E) is farther from the dorsal lip.

   F. Embryo resulting from the foregoing operation. *bp. gr.*, blastoporal groove; *dll.*, dorsolateral lip; *en.*, presumptive entoderm; *hg.*, hind-gut entoderm; *nt.*, neural plate; *pr.*, presumptive chordal projection; *proc.*, proctodeum.

lateral parts of the embryo were depressed and dark grooves marked the sites of the cellophane sheets (*gr.*, fig. 3, F). Several of these eggs developed a rather large bulbous mass at the end of the projection, as is shown in figure 3, F, but this occurred also in several eggs in which strips of presumptive ectoderm had been implanted.

Experiment IV: Superficial implantation of presumptive ectoderm.—In the above-described experiments in which the dorsal lip was isolated by means of presumptive ectoderm the eggs had been subjected to two experimental conditions: the splitting of the marginal zone on both sides of the dorsal lip, and the implantation of presumptive ectoderm into these slits. It was of interest to determine whether one or both of these conditions are necessary to produce the isolation effect, since this would give us some idea of the conditions favorable to normal gastrulation. In 6 of a group of 11 eggs only the *outer* layer of cells of the dorsolateral marginal zones was removed by means of a fine pipette. Strips of presumptive ectoderm were placed upon the raw surfaces and rapidly healed in place. Superficially the eggs looked like the one shown in figure 3, B, but the marginal zone had *not* been cut through. These eggs developed into perfect neurulae. Hence the mere presence of the presumptive ectodermal strips is not sufficient to isolate the dorsal lip.

In the remaining 5 eggs two slits were made completely through the marginal zones on both sides of the dorsal lip, but no presumptive ectoderm was inserted into the slits. These eggs developed into neurulae also; 3 of the specimens were pear-shaped, indicating some difficulty in gastrulation. Gastrulation as a whole was essentially normal, since the yolk masses were engulfed completely and the neural plates were of normal length. Therefore the mere splitting of the marginal zone does not suffice to bring about the isolation of the dorsal lip. Both the splitting of the marginal zone and the implantation of presumptive ectoderm are essential to produce the isolation of the dorsal lip described in experiment III.

Experiment V: Isolation of the dorsal lip by 90° rotation of the dorsolateral lips.—Will presumptive entoderm as well as ectoderm isolate the dorsal lip *in situ?* Attempts to implant narrow strips of entoderm were unsuccessful, since the large cells disintegrated within a short time. Therefore the method of 90° rotation of the dorsolateral marginal zones was used. The ventral edge of the marginal zone adjoining the yolk mass, in the Anura, is presumptive entoderm. If the dorsolateral marginal zones (fig. 5, A) are removed and rotated 90°, one clockwise and the other counterclockwise, the dorsal lip will be flanked on the right and left by presumptive entoderm (fig. 5, B). This was done in two groups of eggs, each composed of 8 early gastrulae. Again the presumptive chordal region extended away from the egg surface and a ring embryo was formed (fig. 5, C). The neural masses (*nt.*) were small but on the whole slightly larger than those formed in eggs which received presumptive ectodermal implants. A peculiarity of these eggs was that the projection extended over the yolk mass in all specimens, as shown in figure 5, C.

In another group of 6 early gastrulae the dorsolateral marginal zones were also rotated (fig. 5, D), but this time in such a manner that the presumptive

entodermal edge was farther removed from the dorsal midline (fig. 5, E) than in the previous experiment. In these operations care was taken that the uppermost cut passed through the line of junction between the roof and floor of the egg, that is, through the presumptive mesoderm of the marginal zone.
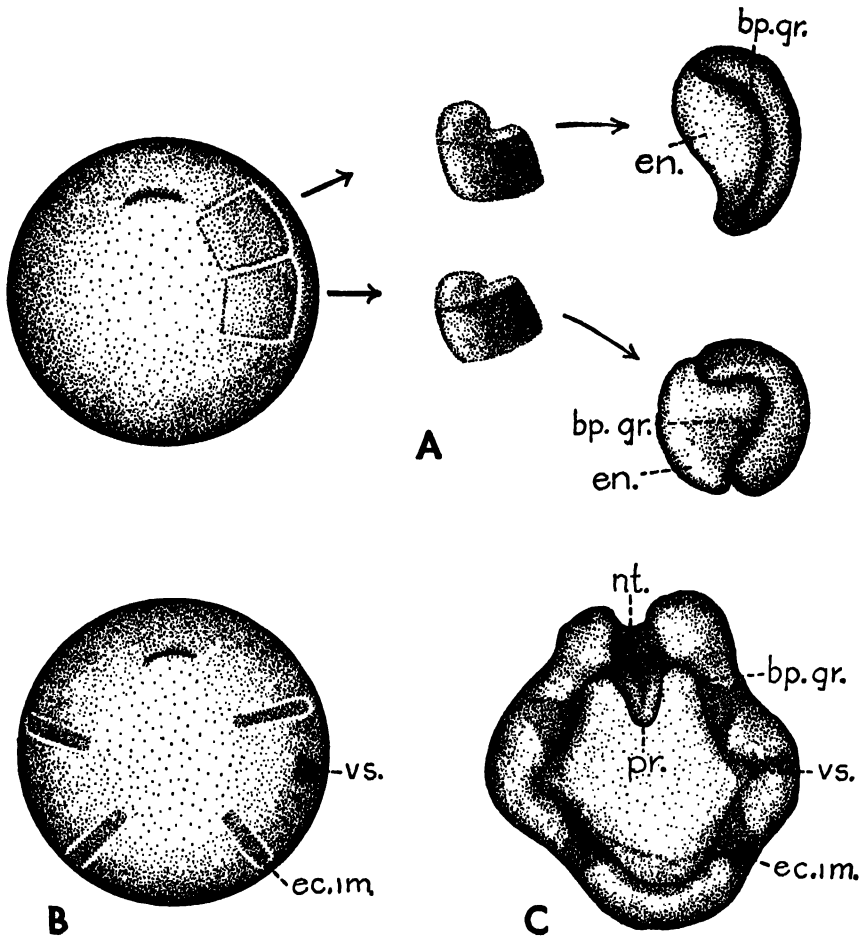
Fig. 6. A. Explantation of dorsolateral and lateral blastoporal lips; at extreme right the explants (enlarged) after 16 hours of culture.
B. Insertion of strips of presumptive ectoderm (*ec. im.*) into slits made through the lateral and ventrolateral marginal zones.
C. Ring embryo resulting from the foregoing operation. *bp. gr.*, blastoporal groove; *ec. im.*, presumptive ectodermal implant; *en.*, presumptive entoderm; *nt.*, neural tissue; *pr.*, presumptive chorda projection; *vs.*, vital-stain mark.

None of these eggs developed projections, but gastrulation was seriously impeded (fig. 5, F). Large amounts of yolk remained visible externally and the neural plates were about one-third to one-fourth as long as normal plates. The best specimen obtained is shown in figure 5, F; in the remaining 5 eggs the neural plates were somewhat shorter. The presumptive entoderm of the dorsolateral marginal zone is thus capable of isolating the dorsal lip *in situ*. But

when the presumptive entoderm is farther from the midline and the dorsal lip is flanked by presumptive mesoderm, gastrulation is improved to some degree.

Experiment VI: The lateral marginal zone explanted and isolated *in situ*.— Since it appeared from the foregoing experiments that the normal behavior of the dorsal lip was in some way dependent upon its connection with lateral regions of the marginal zone, it was desirable to see what movements these lateral regions can carry out when they are isolated. Explants were made from two portions of the marginal zone (fig. 6, A). Furthermore, strips of presumptive ectoderm were inserted in slits made through the lateral and ventrolateral marginal zones (fig. 6, B). The explants developed a clear blastoporal groove (*bp. gr.*, fig. 6, A) and the blastoporal lip underwent some degree of involution. The explants taken from the more dorsal position tended to elongate more than those from the lateral position (fig. 6, A). But it is clear, if one takes into consideration the curvature of the blastoporal grooves, that the blastoporal lips have elongated in both types of explants. Even the small amount of entoderm included in these explants was not covered by marginal zone material.

In isolation *in situ* by presumptive ectoderm (fig. 6, C) deep blastoporal grooves developed, and vital-stain marks placed on the lateral marginal zones were turned inward (*vs.*). There was also apparently a tendency for the isolated lateral marginal zones to contract, as is suggested by their baylike indentation. But the lateral marginal material did not converge dorsalward, nor did it constrict so as to cover the entodermal mass. A short neural plate was formed (*nt.*, fig. 6, C) and behind it a short, blunt projection (*pr*). The presumptive ectodermal implants (*ec. im.*) were partly involuted and stretched in a dorsoventral direction.

Experiment VII: The dorsal lip transplanted into the presumptive ectodermal region.—The results of this type of operation are well known from work on several European Amphibia (Mangold, 1923; Spemann, 1931; Lehmann, 1932; Töndury, 1936; Vintemberger, 1938*b*). Our work on *Hyla regilla* conforms to these previous descriptions, and hence a brief account will suffice. In a group of 11 early gastrulae the dorsal lip, including a small amount of sub-blastoporal material, was excised (fig. 7, A) and then transplanted near the animal pole (fig. 7, B). In some the implanted dorsal lip was oriented so that its presumptive entodermal edge was directed toward the host's ventral lip (as in fig. 7, B); in others the implant was placed in the reverse orientation. It is important in such experiments that the implants be observed about one-half hour after the operation in order to make certain that the edges have healed smoothly with the surrounding tissue. If healing is incomplete the implant may sink under the adjacent presumptive ectoderm and later convey the impression that excellent invagination has taken place. The blastoporal groove of the implant extended under the host's surface to form a deep pocket (*bp.*, fig. 7, C), but the presumptive chordal region projected away from the surface (*pr.*). A small mass of neural tissue was formed at the base of the projection (*nt.*, fig. 7, C). While it is true, then, that the
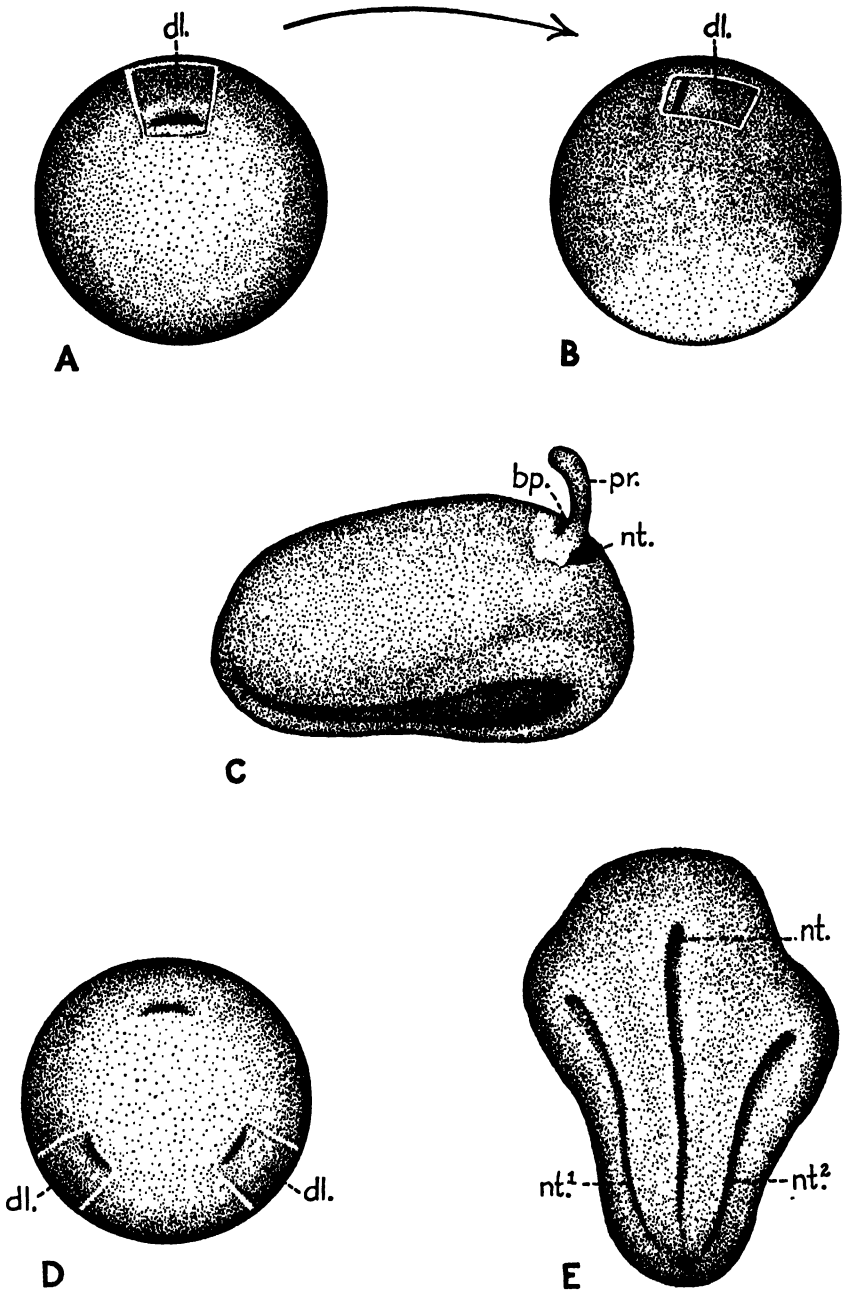
Fig. 7. A. B. Transplantation of dorsal lip (*dl.*) from one egg (A) into presumptive ectodermal region of another egg (B).

C. The implant has developed a blastoporal pocket (*bp.*) on one side of the presumptive chordal projection (*pr.*), and a patch of neural tissue (*nt.*) on the opposite side.

D. Implantation of two dorsal lips (*dl.*) into the ventrolateral marginal zones.

E. Embryo resulting from the foregoing operation (D, above). *nt.*, primary neural plate; *nt.*[1] and *nt.*[2], secondary neural plates. See figure 5, plate 7, for cross section.

dorsal lip has a tendency to sink below the surface, this statement applies to the circumblastoporal region rather than to the presumptive chordal region. This distinction was also noted by Vintemberger (1938*b*) in *Rana fusca*.

Experiment VIII: Dorsal lips transplanted into the ventrolateral marginal zones.—The dorsal lip stretches, involutes, and invaginates with great regularity in this position (Lehmann, 1932; Vintemberger, 1938*a*; and others). This was confirmed in *Hyla regilla*. Of 11 dorsal lips so implanted, 9 passed under the host's presumptive ectoderm, in which secondary neural plates about one-half to two-thirds the normal length were induced. In the 2 remaining specimens the transplants did not fuse evenly with surrounding tissue and developed into elongate projections.

Since the preceding experiments indicate that the presence of the dorsal lip promotes dorsalward convergence, it appeared possible that the convergence might be prevented if *two* dorsal lips were implanted into the ventrolateral marginal zones; for the two implants should tend to cause a convergence toward themselves and so compete with the host's own dorsal lip. Hence each of 4 early gastrulae received two dorsal lip implants in its ventrolateral marginal zones (fig. 7, D). All three dorsal lips now present in every egg involuted and invaginated excellently so that three long neural plates were induced (fig. 7, E). The two implants did not prevent normal dorsalward convergence; in fact, they were themselves carried dorsalward during gastrulation, so that the two secondary neural plates (*nt.*[1] and *nt.*[2], fig. 7, E) developed close to the primary plate. The strong dorsalward convergence which occurred in these eggs is shown more clearly in sections (pl. 1, fig. 5). The three neural plates are so close together as to appear fused at their edges. Three notochords are present, but only a single archenteric cavity. While, therefore, the dorsal lips show excellent capacities for involution and invagination when they are located in the marginal zone, their ability to promote convergence toward themselves is relatively weak as compared with the dorsal lip in its *normal* location.

MUTUAL EFFECTS EXERTED BETWEEN VARIOUS REGIONS OF THE MARGINAL ZONE

The foregoing experiments indicate that certain of the movements of gastrulation are not autonomous or inherent in the specific materials which normally carry out these movements. We may designate these movements as the *correlative movements* of gastrulation; they include invagination of the presumptive chordal region, dorsalward convergence of the marginal zones, and constriction of the blastoporal lips over the yolk mass. The experiments further suggest that material of the presumptive chorda acquires its capacity for invagination from contact with other regions of the marginal zone, and that the latter acquire their capacities for convergence and constriction from the dorsal lip. If this hypothesis holds, it should be possible to repeat the foregoing experiments and induce the correlative movements of gastrulation by including appropriate portions of the marginal zone.

Experiment IX: The dorsal lip explanted together with lateral portions
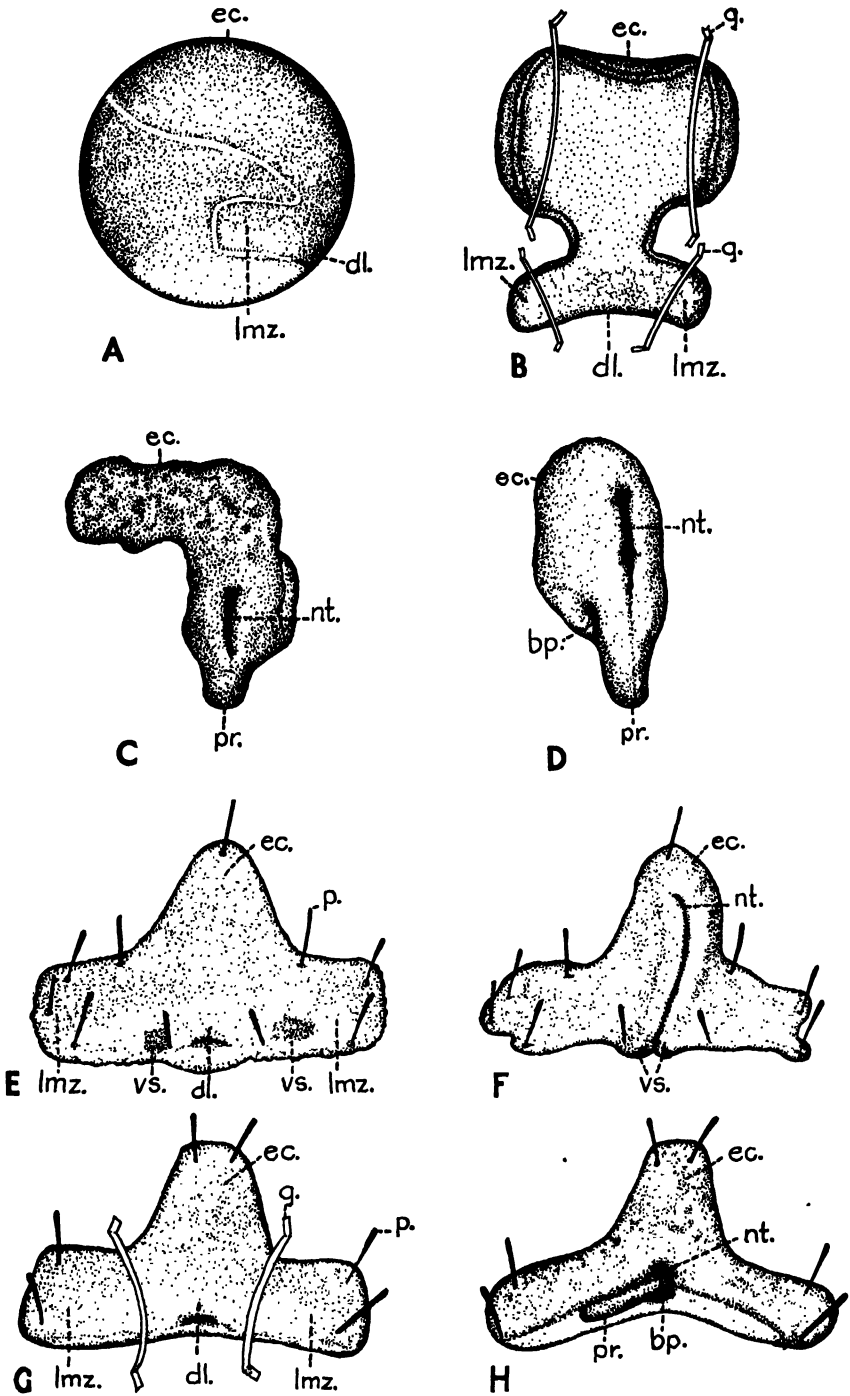
FIG. 8

(For explanation of figure 8 see foot of following page.)

of the marginal zone.—In two groups, each composed of 12 eggs, wide strips including the dorsal lip and adjacent portions of the dorsolateral marginal zone were excised, together with a large amount of presumptive ectoderm (fig. 8, A and B). This is the counterpart of experiment II. The tin-foil girders did not wholly prevent curling of the presumptive ectoderm, although the curling was delayed as in experiment II. Four hours after the operation, the lateral parts of the marginal zone (*lmz.*, fig. 8, B) had contracted toward the central (dorsal lip) part, thus escaping from under the two small girders. The presumptive ectoderm also escaped from the girders and subsequently became a solid, wrinkled mass (*ec.*, fig. 8, C) or formed a smooth layer surrounding the main body of the explant (fig. 8, D). There was always present an elongate neural mass (*nt.*), and behind it a blunt, gray-brown projection (*pr.*). The previous failure of the presumptive chorda to turn inward (in experiment II) might conceivably have been due to mechanical impedence by the curling presumptive ectoderm, but this becomes improbable in view of the present experiments. The degree of invagination was clearly far short of that in normal eggs, for the neural plates were never more than one-third normal in length, and some of the marginal material was not invaginated, as is shown by the projecting masses. A transverse section through the specimen shown in figure 8, D, is given in plate 1, figure 4. The presumptive ectoderm had curled to the greatest extent in this specimen, and hence one might expect a maximal degree of resistance to the invagination of the presumptive chorda. However, about one-third of the presumptive chorda was invaginated (*ch.*, pl. 1, fig. 4); and the chorda was flanked by two mesodermal masses (*m.*). Around the latter there is a layer which is probably entodermal (*en.*), as is indicated by the large yolk platelets and indistinct cell boundaries. In more anterior sections the explant is surrounded by a layer of presumptive ectoderm.

In two other groups, each composed of 9 eggs, still more of the marginal zone was included in the explants, and an improved method was employed to prevent curling of its lateral portions (fig. 8, E). The explants were laid upon their inner, unpigmented surfaces and fastened to the wax substrate with fine black-glass pins (*p.*). Vital-stain marks (*vs.*) were placed on both sides of the dorsal lip so that the movements of materials might be followed better. Every explant carried out excellent invagination, and long neural plates were formed (*nt.*, fig. 8, F). The vital-stain marks converged toward the midline, moving around the near-by pins, and were drawn out into long, thin streaks

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Fig. 8. A. Removal of the dorsal lip (*dl.*) together with lateral marginal zone (*lmz.*) and much presumptive ectoderm (*ec.*).

B. The explant fastened to the substrate by means of tinfoil girders.

C. D. Two variations in the development of the explants after 16 hours of culture.

E. G. Explants composed of the dorsal lip (*dl.*) and more of the lateral marginal zone than in figure 8, A and B, showing two methods employed to fasten the explants to the substrate by means of glass pins (*p.*) and tinfoil girders (*g.*).

F. H. Two variations in the development of the explants after 16 hours of culture.

bp., blastopore; *dl.*, dorsal lip; *ec.*, ectoderm; *g.*, tinfoil girder; *lmz.*, lateral marginal zone; *nt.*, neural tissue; *p.*, glass pin; *pr.*, presumptive chorda projection; *vs.*, vital-stain mark.
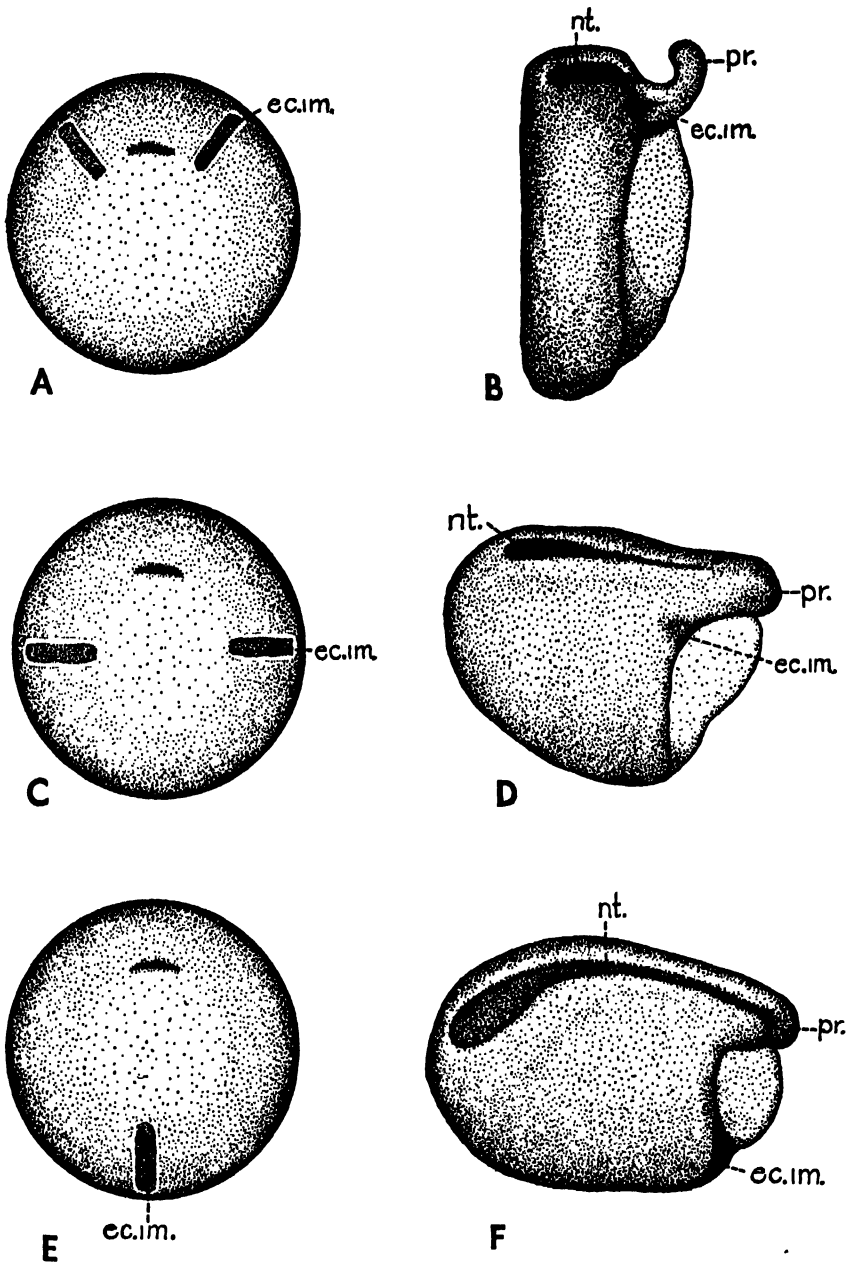
Fig. 9. A. C. E. Implantation of strips of presumptive ectoderm into slits cut through the marginal zone at various distances from the dorsal lip.

B. D. F. Neurulae resulting from the foregoing operations, A, C, E, respectively. *ec. im.*, ectodermal implant; *nt.*, neural plate; *pr.*, projection.

on the underside of the explants; only their extreme posterior tips were visible from dorsal view (*vs.*, fig. 8, F). It is indeed remarkable that such extensive invagination could take place despite the fact that the material of the lateral marginal zone remained fastened to the substrate throughout the entire gastrulation period.

In another group of 5 eggs, in addition to the pins 2 girders were placed tightly over the dorsolateral marginal zones of the explants in an attempt to check dorsalward convergence and involution (fig. 8, G). In 3 of these explants, results were the same as those shown in figure 8, F. In the other 2, invagination of the dorsal lip was prevented. Fingerlike projections developed, but they were attached to the adjacent marginal zone in bas-relief (*pr.*, fig. 8, H). Only a tiny neural mass was present (*nt.*), but the blastoporal pocket was clear and deep (*bp.*).

We see, therefore, that the presumptive chordal material shows strong capacities for invagination in explants, *provided* it is in contact with more laterally situated portions of the marginal zone.

Experiment X : Various amounts of marginal zone isolated *in situ* by presumptive ectoderm.—This is the counterpart of experiment III. In the present experiments the strips of presumptive ectoderm were implanted into slits cut through the marginal zone at various distances from the dorsal midline (fig. 9, A, C, E). In this way the dorsal lip plus various amounts of lateral marginal zone was isolated *in situ*. When approximately one-fourth of the dorsal marginal zone was thus isolated (fig. 9, A), the eggs developed into ring embryos (fig. 9, B) ; but the neural plate (*nt.*) was clearly longer, and the projection (*pr.*) shorter, than in eggs in which only the dorsal lip proper was isolated (cf. fig. 3, A–D). The blastoporal pocket was also deeper, as was shown by dissection, and constriction was somewhat improved since the blastoporal lips extended farther up the sides of the yolk mass. The dorsal marginal zone included between these strips of presumptive ectoderm contains most of the neural inductor, as was shown by implantation of pieces of the marginal zone into the blastocoele (Schechtman, 1938). The small neural plate in the present specimens is not, therefore, the result of any deficiency of neural inductor; a piece of dorsal marginal zone of this size in the blastocoele commonly induces a secondary neural plate two-thirds to three-fourths the length of the normal plate.

When the upper half of the marginal zone was isolated from the lower half (fig. 9, C), gastrulation was greatly improved (fig. 9, D). The neural plate was about half the normal length, and much less of the yolk mass was visible. Some of the presumptive mesoderm immediately above the implants did not invaginate, as is shown by the presence of a blunt projection (*pr.*), but it was nevertheless carried to a dorsal position. The most complete gastrulation was seen in specimens in which the marginal zone was split in the midventral line (fig. 9, E). The neural plate was here of about normal length and much of the yolk mass was engulfed (fig. 9, F).

If this series of eggs is compared with the explants described in experiment IX (fig. 8), it is apparent that the more marginal zone material there is in

continuity with the dorsal lip, the greater is the degree of invagination and constriction.

Experiment XI : The dorsal lip, together with lateral parts of the marginal zone, transplanted into the presumptive ectodermal region.—This is the counterpart of experiment VII. In a total of 8 eggs the dorsal third of the marginal zone was transplanted into the presumptive ectodermal region, as shown in figure 10, A and B. Three of the eggs had to be discarded because the trans-
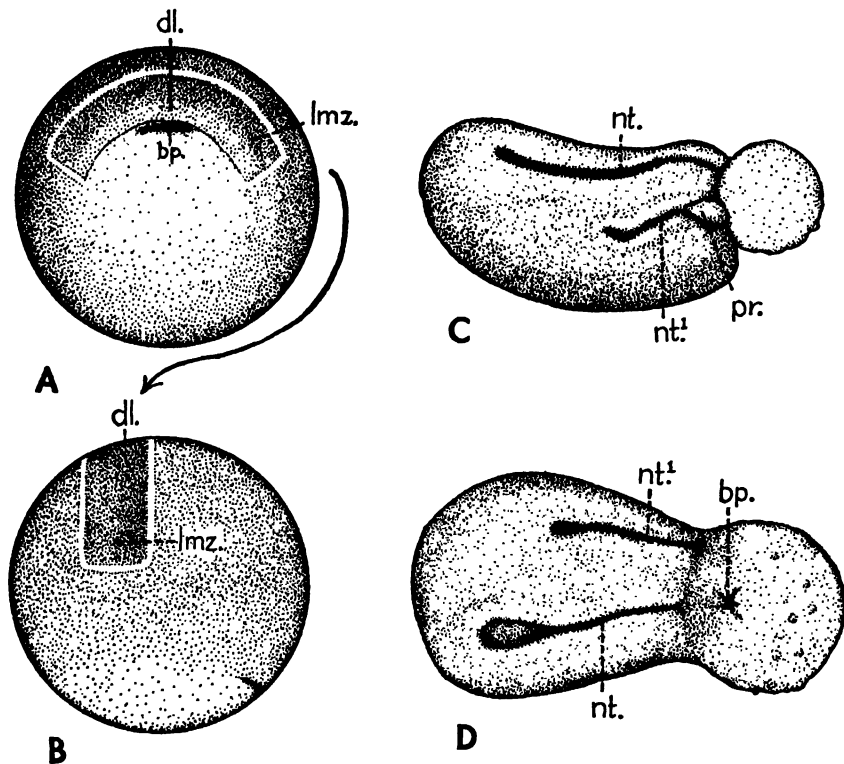
Fig. 10. A. B. Transplantation of dorsal lip (*dl.*) together with lateral marginal zone material (*lmz.*) from one egg (A) into the presumptive ectodermal region of another (B).
    C. D. Two types of neurulae resulting from the foregoing operation. *bp.*, blastopore; *lmz.*, lateral marginal zone; *nt.* and *nt.*[1], primary and secondary neural plates; *pr.*, projection.

plants did not fuse readily but curled and took up irregular positions. In the remaining 5 the transplants carried out various degrees of invagination, as is shown by the lengths attained by the secondary neural plates (*nt.*[1], fig. 10, C and D). In none was there an elongate projection such as is characteristic of pure dorsal-lip transplants (cf. fig. 7, C). In 4 of the 5 eggs there were short projections (*pr.*) such as that shown in figure 10, C. In one (fig. 10, D) there was no projection at all, but this egg evidently had acquired a tend ncy toward exogastrulation, for the blastoporal lips had vanished and the blastoporal pit (*bp.*) lay on the yolk mass.

From these experiments it seems improbable that the failure of the pre-

sumptive chordal material to invaginate is to be explained on the basis of some kind of mechanical hindrance present in the presumptive ectodermal region. As in explants and dorsal lips isolated *in situ,* we have only to add some lateral marginal zone in order to bring about invagination. There is, of course, always the possibility that some degree of mechanical hindrance is involved, although this is as yet a hypothesis which has not been proved experimentally. However, irrespective of whether the supposed hindrance actually exists, the presumptive chorda acquires the capacity to invaginate in the presence of lateral portions of the marginal zone.

NONSPECIFICITY OF VARIOUS REGIONS OF THE MARGINAL ZONE IN PROMOTING GASTRULATION

In the foregoing I believe I have demonstrated that normal gastrulation requires mutual interactions among various regions of the marginal zone. Thus, although the dorsal lip is inherently capable of extension, the invagination of its presumptive chordal portion depends upon an auxiliary effect exerted by the lateral marginal zones. Now, to understand development in general it is necessary to ascertain the degree of localization of the various developmental capacities of the egg. Hence we may ask with respect to the auxiliary effects, Are they limited to particular regions of the marginal zone, or may one region substitute for another in the movements of gastrulations?

Experiment XII: The dorsal lip replaced by ventral lip.—In 7 early gastrulae the dorsal lips were excised and replaced by pieces of ventral lip from other early gastrulae (fig. 11, A and B). The transplanted ventral lip was arranged in normal orientation, its presumptive entodermal edge directed toward the entodermal mass of the host. Gastrulation was excellent (fig. 11, C). Since the dorsolateral marginal zones had been separated by the implant, two distinct neural tubes developed (*nf.,* fig. 11, C), separated by a smooth expanse of nonneural tissue (*vlt.*) derived from the implant. Some of the eggs were allowed to develop to the tail-bud stage so that a higher degree of histological differentiation might be obtained, and were then sectioned. At this time the existence of two distinct neural tubes was confirmed; under each tube there was a length of chorda. An undifferentiated mesodermal mass derived from the transplanted ventral lip lay between the two neural tubes. It is thus obvious that the ventral lip had not replaced the chorda in a material sense; it had, in fact, split the axial organs into two separate masses. But, so far as the movements of gastrulation were concerned, the ventral lip had effectively performed the function of the dorsal lip since the eggs carried out a dorsalward convergence and the blastoporal lip constricted over the yolk mass. These experiments confirm similar ones carried out by Bautzmann (1933) on *Pleurodeles* and *Limnodynastes.* Töndury (1936), working on *Triton alpestris,* found that the ventral lip placed in the dorsal lip position was transformed into chorda; his results are thus in opposition to those of Bautzmann and the present work on *Hyla.*

The donor gastrulae, from which the ventral lip had been removed, developed into excellent neurulae the only defect of which was a slightly protrud-
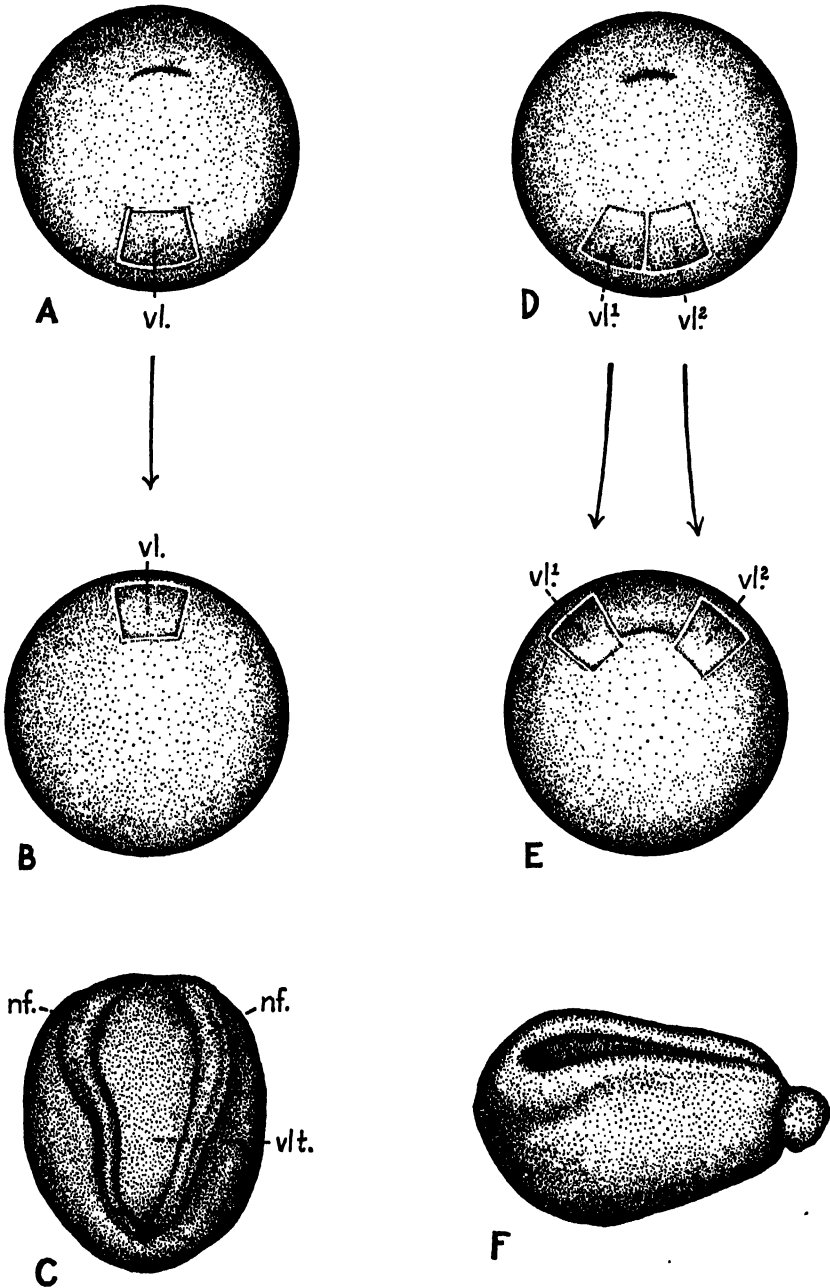
Fig. 11. A. B. Transplantation of ventral marginal zone (*vl.*) from one egg (A) to the dorsal lip position of another egg (B).

C. Neurula resulting from this operation. The two neural folds (*nf.*) are separated by a layer of nonneural tissue (*vlt.*) derived from the transplant.

D. E. Transplantation of ventral marginal zones (*vl.*[1] and *vl.*[2]) from one egg (D) to the positions of the dorsolateral lips of another egg (E).

F. Neurula, almost normal, resulting from the foregoing operation.

ing yolk mass at the posterior end. This stands in marked contrast to eggs lacking the dorsal lip (cf. fig. 1, F and G).

Experiment XIII: The dorsolateral lips replaced by ventral lip.—May the auxiliary effects of the dorsolateral lips be exerted by other regions of the marginal zone? In 6 early gastrulae both dorsolateral lips were excised and replaced by pieces of the ventral lip (fig. 11, D and E). The eggs gastrulated well, but some difficulty was apparently involved since in all specimens part of the yolk mass remained visible externally (fig. 11, F). It may be concluded from these experiments that the auxiliary capacities are not limited to specific regions of the marginal zone; the ventral marginal zone may perform the work of the dorsal or dorsolateral marginal zones if placed in their position.

DISCUSSION

THE NECESSITY FOR AUXILIARY EFFECTS IN EXPLAINING THE MOVEMENTS OF GASTRULATION

Born's early theory of gastrulation (1884) postulated that the movements of invagination were the result of growth processes created by the expanding animal hemisphere when it meets the resistance of the "inert" yolk mass. This theory had to be discarded as a result of Vogt's experiments (1922) in which he removed a large amount of presumptive ectoderm in the early blastula stage and prevented the downward movement (epiboly) of the animal hemisphere. Nevertheless, a pit developed in the vegetative region at or near the site of the normal blastoporal groove, and the marginal zone constricted and formed a deep furrow equivalent to the circular blastoporal groove. Although Holtfreter (1933, p. 692) has arrived at a somewhat different interpretation, he has confirmed Vogt's conclusion that the movements of gastrulation in exogastrulae occur independently of the epiboly of the presumptive ectoderm. Recent work on an invertebrate egg (*Dendraster excentricus*) has led Moore and Burt (1939) to a similar conclusion, namely, that the invagination of the lower part of the egg, the entodermal plate, is brought about by forces exerted within this material rather than by pressure from without.

Although Kopsch (1895, cited by Vogt, 1929, p. 403, and by Roux, 1902, p. 608), on the basis of photographs of gastrulating eggs, argued for the existence of active streaming movements in the entoderm, convincing evidence of autonomous movements came from transplantation and explantation experiments (reviewed in Spemann, 1938). These experiments culminated in the autonomy theory as expounded by Spemann and quoted previously (p. 1). But several observations do not fit well into the autonomy theory. The failure of part of the dorsal lip to invaginate after transplantation into the presumptive ectoderm led Lehmann (1932) to postulate the existence of assisting factors. He suggested that the dorsal lip may be *pressed* inward by the marginal material adjacent to it (p. 603). Spemann (1938), in describing the results of dorsal lips transplanted into presumptive ectoderm and into ventral blastoporal lips, remarks: "... in the animal region the invagination finds least assistance (hence the excrescences and protuberances described before and

observed by various authors). . . . the nearer to the blastopore, the easier becomes invagination" (p. 152). Vintemberger (1938a, b) found that all parts of the dorsal lip do not possess equal capacities for invagination; the regions close to the early blastoporal groove have a strong tendency to invaginate, but the presumptive chordal region is deficient in this respect. Vintemberger saw the need for postulating auxiliary effects, for he states that the presumptive head mesoderm is sometimes capable of effecting the invagination of the presumptive chordal region (1938a, p. 435). Waddington (1940, p. 110) has made a similar suggestion. The experiments reported in the present paper do not support this view of the source of the auxiliary effects, since the presence of the circumblastoporal region did not suffice to bring about invagination of the presumptive chorda under diverse experimental conditions: when the dorsal lip was explanted, or isolated *in situ*, or transplanted into the presumptive ectodermal region. Dalcq (1940) showed that after the excision of the dorsal lip the marginal zone fails to converge dorsalward. He suggested that the presumptive chorda must control this movement.

Now, Holtfreter (1939b) found that explants of *pure* presumptive mesoderm are capable of only very limited mass movements although they differentiate well in a histological sense. But if other organ-forming materials—presumptive ectoderm or entoderm—are added to the pure presumptive mesoderm, the capacity to carry out movements is increased. Holtfreter concludes that mutual influences must be exerted between the parts. However, he believes that these mutual influences consist of substrate effects: the organ-forming materials have inherent capacities to perform the movements of gastrulation, but require a substrate upon which to carry out these movements.

In the present experiments an attempt was made to provide the conditions which Holtfreter considers essential. Thus, the dorsal lip was provided with abundance of adjacent substrate (the inner surface of the animal hemisphere) in experiments II and III. But the presumptive chordal region exhibited little or no capacity for invagination. The circumblastoporal material behaved differently: it continued to invaginate, whether in the explants, in the transplants, or *in situ*. Similar considerations apply to eggs lacking the dorsal lip. Such eggs, of course, contain numerous organ-forming anlagen and large expanses of surface. Yet dorsalward convergence and constriction over the yolk mass did not take place. This suggests that the mere presence of substrate and of a multiplicity of anlagen does not provide all the conditions necessary for the movements of gastrulation.

It must be emphasized that no denial is here made of the existence and importance of autonomous movements in gastrulation. All evidence available at present indicates that the extension or stretching of the marginal zone, the depression of the egg surface to form a blastoporal groove, and the inward migration of lateral and ventral marginal zones are autonomous movements. Furthermore, Holtfreter's (1939a, b) observations on the role of substrates and on the changing affinity between various parts of the embryo seem to offer plausible explanations of how the autonomous movements are directed and brought to realization.

The Nature of the Auxiliary Effects

The movements not accounted for by autonomous forces, substrate effects, and tissue affinity have been designated as correlative, in the sense that the regions of the gastrula which normally perform these movements are dependent upon aid supplied by other regions. A significant point is the nature of the aid rendered, and we may obtain some indication of this by comparing the behavior of the interacting parts.

1. The invagination of the presumptive chordal region.—We have seen that the presumptive chorda material does not invaginate even though it retains continuity with the presumptive pharyngeal entoderm and with the mesoderm in the interior of the dorsal lip. Both regions were present in the various explanted and transplanted dorsal lips, as well as in those isolated *in situ;* yet the presumptive chorda did not invaginate. But invagination did take place if the dorsal lip retained continuity with the lateral marginal zones. We have seen also that the lateral marginal zones have a strongly developed capacity for involution and invagination, whether explanted, transplanted (Mangold, 1923), or isolated *in situ*. From Vogt's (1929) work with vital staining we know that the presumptive pharyngeal entoderm begins to invaginate first, for the earliest in-sinking of the surface to form a blastoporal groove occurs in this material. By the time the presumptive chordal region arrives at the brink of the dorsal lip the dorsolateral marginal zones are actively moving inward. These experiments suggest that the motive force for the invagination of the presumptive chorda may be supplied by a tension or pull exerted by the invagination and involution of the dorsolateral and lateral marginal zones. The magnitude of these movements is great enough to turn in two dorsal lips (experiment VIII) or several strips of presumptive ectoderm (experiment VI). Lehmann's (1932) suggestion of a *pressure* applied against the flanks of the dorsal lip by the lateral lips seems untenable, for on this basis we should expect the lateral marginal zones to press toward the midline when the dorsal lip is excised, and this does not occur.

Waddington (1940) has suggested that the invagination of the presumptive mesoderm "seems likely to depend essentially on a single step; if the stream of elongating presumptive mesoderm is once coaxed inward, it will continue to roll around the blastopore lip" (p. 110). This appears unlikely, at least so far as the presumptive chorda is concerned, for the degree of invagination is roughly proportional to the amount of lateral marginal zone in contact with the dorsal lip (experiments IX and X). The situation seems rather to involve a *progressive* auxiliary effect.

2. The convergence-promoting effect.—The dorsalward convergence of successive dorsoventral levels of the marginal zone depends upon the continuity of each level with all the levels of the marginal zone above it. This was shown in experiment X, in which the portions of the marginal zone above (dorsal to) the implanted presumptive ectoderm moved dorsalward. Now this is just what one would expect if convergence were indeed the expression of a *pull* or *tension* exerted by the dorsal lip. The question then arises, Is there evidence

that the stretching of the dorsal lip is powerful enough to pull or drag a large amount of material after it? A number of observations indicate clearly that the stretching is a powerful movement. It occurs under the most varied conditions: in explants, in transplants made into the presumptive ectodermal region, in implants into the blastocoele, and after isolation of the dorsal lip *in situ*. The extension, moreover, does not require attachment to a substrate, as was shown by Holtfreter (1939*b*, p. 267), and as is shown also in the present experiments. It seems clear that it is not the invagination, but merely the extension and simultaneous narrowing of the dorsal lip, that accomplishes convergence, for even when the dorsal lip merely stretches but does not invaginate, as in exogastrulae, the laterally situated presumptive mesoderm converges toward the midline (Holtfreter, 1933).

3. The constriction-promoting effect.—The constriction of the blastoporal lips over the yolk depends upon continuity between the dorsal lip and the more laterally situated portions of the marginal zone. The degree of constriction increases as larger amounts of lateral marginal zone are left in contact with the dorsal lip (experiment X). Constriction is thus dependent upon the same conditions as promote invagination of the presumptive chorda and dorsalward convergence of the lateral lip materials. We may correlate constriction, invagination of the presumptive chordal material, and convergence into the following picture of the mechanism of gastrulation. Gastrulation begins with autonomous movements: the in-sinking of the presumptive pharyngeal entoderm, the stretching of the marginal zone toward the blastoporal groove, the forward migration of the internally situated marginal material along the underside of the animal hemisphere. As the stretching presumptive chordal region comes to the edge of the blastoporal lip, it is progressively carried under by the invagination and involution of the adjacent portions of the marginal zones. This insures that the presumptive chorda will stretch in the right direction—toward the interior (or anterior of the embryo). Now the presumptive chorda is in a position to exert a double effect by means of its extension, for it will not only pull the lateral marginal zones dorsalward, but will also carry them forward in a dorsal position. Meanwhile the blastoporal lips are constricting, since there is a progressive withdrawal of marginal zone material by the process of dorsalward convergence somewhat as the mouth of a purse is constricted when the purse string is pulled. The tendency to constriction is augmented further by the forward migration of the *internal* portions of the marginal zone, for this movement also tends to withdraw material from the region of the blastoporal lips (Vogt, 1929).

Now, on this basis constriction is to a large degree the consequence of dorsalward convergence, brought about by the extension and narrowing of the presumptive chorda material, which in turn is invaginated by the action of the lateral marginal zone. However, Spemann (1938, p. 102) describes constrictions which apparently occurred independently of dorsalward convergence. Several figures based on the exogastrulation experiments of Vogt are given, showing an egg constricting to form a dumbbell-shaped mass (*ibid.*, fig. 56). Holtfreter (1933) has studied the movements of such exogastrulae by means

of vital-stain marks and has shown that the constriction of these exogastrulae is accompanied by the same movements as occur in normal gastrulation, but in the reverse direction. The presumptive chorda material elongates over the yolk mass, and the lateral portions of the marginal zone converge dorsalward, that is, toward the presumptive chorda. The fundamental causes of exogastrulation are unknown, but the present results suggest that they are to be sought in the lateral marginal zones which are unable to exert their invagination-promoting effects upon the presumptive chorda material.

It has not, so far as I am aware, been shown that constriction of the blastoporal lips occurs in the absence of extension of the presumptive chorda and dorsalward convergence of the marginal zone. Even under the most abnormal experimental conditions these three movements are apparently always associated. A remarkable example is seen in the work of Eakin (1939), who filled the blastocoele of the late blastula or early gastrula with gelatin. Neither the yolk mass nor the marginal zone could move into the blastocoele; nevertheless the bastoporal lips constricted over the yolk mass, and the constriction was accompanied by convergence of the marginal zones and extension of the presumptive chorda.

CAUSES OF INCOMPLETE CLOSURE OF THE BLASTOPORE

The failure of the blastoporal lips to constrict over the presumptive entoderm so that part of the yolk mass remains visible in later development as a persistent yolk plug is one of the most commonly observed anomalies of amphibian development. The defect may vary in degree from a small persistent yolk plug to the extreme condition seen in ring embryos, in which almost the entire yolk mass is exposed (*spina bifida,* Hertwig; *asyntaxia medullaris,* Roux). These anomalies have been observed in nature (Hertwig, 1906; Bergel, 1927) and are frequently encountered in the laboratory. They may be produced by the most diverse experimental conditions: exposure to inorganic salt solutions (Hertwig, 1906; Jenkinson, 1906; Bellamy, 1919), dinitrophenol (Dawson, 1938), ultraviolet irradiation (Baldwin, 1915), abnormally high temperatures (Hertwig, 1906), or by aging unfertilized eggs (Zorzoli and Rugh, 1942). A variety of other treatments is given by Hertwig (1906) and by Dawson (1938).

Jenkinson (1906, 1914) advanced the hypothesis that this defect was primarily a result of injury to the yolk mass rendering it later unable to move into the interior of the egg. The yolk mass, in Jenkinson's opinion, is highly susceptible to adverse environmental conditions because of its high yolk content and the paucity of active cytoplasm. However, it has never been shown that the yolk mass is an especially susceptible region of the egg. Bellamy (1919) found that the dorsal lip and the region around the animal pole are sites of maximal susceptibility to a number of toxic substances. Dalcq (1940) showed that ring embryos could be produced by removing the dorsal lip. Dalcq's finding has been verified in the present paper, and furthermore it has been shown that the degree of the defect may be controlled experimentally by surgical operation of the marginal zone. Pasteels (1940b) is of the opinion

that some cases of *spina bifida* (produced by centrifuging) arise from defects of the dorsal lip rather than of the yolk mass. It seems clear that persistence of the yolk mass may be caused by defects of the marginal zone. Moreover, the data of the present paper (particularly experiment X) indicate that the magnitude of this developmental anomaly varies with the locus of the injury— the closer the defects are to the dorsal lip, the greater the amount of uninvaginated entoderm.

It must not, however, be concluded that persistence of the yolk plug always indicates defects in the marginal zone alone. Roux (cited by Hertwig, 1906, p. 973) produced this anomaly by pricking the eggs with a needle near the vegetative pole or in the marginal zone. Baldwin (1915) found that it could be produced by irradiating the lower surface of the yolk mass to the exclusion of the marginal zone. Baronofsky and Schechtman (1938) produced large persistent yolk plugs by ultraviolet irradiation of the yolk mass or of the marginal zone. It is therefore possible that when the entire egg is subjected to adverse conditions the failure of the blastoporal lips to constrict over the yolk mass may result from injury to the yolk mass, to the marginal zone, or to both.

The Isolating Effect Produced by Presumptive Ectoderm and Entoderm

An important consideration in connection with the present experiments is the manner in which presumptive ectoderm and presumptive entoderm exert their isolating effect upon the dorsal lip. Both these materials have a tendency to increase their areal extent during gastrulation (Vogt, 1939; Holtfreter, 1933, 1939*a*). This spreading tendency may well be an important factor in producing isolation by means of implants, for the force normally exerted by one region of the marginal zone upon another would be expended in merely stretching the implants. However, strips of cellophane also isolate the dorsal lip (experiment III), since they break the continuity of the marginal zone completely and thus prevent one region from helping the other.

Spemann and Geinitz (1927), Mangold (1923), and others transplanted presumptive ectoderm into the dorsal and lateral blastoporal lips and yet did not obtain the isolating effects here described. A careful examination of these experiments reveals the reasons for the apparent discrepancy. Spemann and Geinitz implanted round pieces of presumptive ectoderm into the dorsal lip; an essentially normal gastrulation followed, and the implant was carried inward. Töndury (1936) excised the dorsal lip and the adjacent presumptive ectoderm, rotated the mass 180°, and implanted it into the same egg so that the presumptive ectoderm occupied the dorsal-lip position. Here, too, gastrulation was excellent. In such experiments it is to be expected that the implanted presumptive ectoderm would be invaginated, for the cut edges rapidly fuse and would therefore be subject to the invagination force of the lateral marginal zone, as is the normal dorsal lip. Meanwhile the implanted presumptive ectoderm is being transformed to chorda and somite material, as Spemann and Geinitz and Töndury have shown. Hence there would follow the normal action of the dorsal lip in promoting dorsalward convergence and constriction by its

elongation. Mangold (1923) carried out experiments like those of Spemann and Geinitz, but also implanted presumptive ectoderm into the lateral marginal zone. Gastrulation was often so greatly impeded that *spina bifida* embryos resulted. Sometimes it proceeded more normally, although with some difficulty, and the implants were invaginated. Now, Mangold implanted a piece of presumptive ectoderm on one side only, so that the other side of the marginal zone was free to act in a normal manner. Moreover, the implants used by Spemann and Geinitz and by Mangold were comparatively small, and it seems improbable that the marginal zone was split through completely. It is shown above (experiment IV) that presumptive ectoderm inserted *superficially* into the marginal zone does not exert an isolating effect. I therefore find no reason to conclude that the results herein described are contradictory to previous work, or that *Hyla* presents a special case.

SUMMARY

1. Most of the recent work on the mechanism of gastrulation in Amphibia has stressed the autonomous nature of the various movements. This has led to the view that the orderly sequence of movements depends upon an intricate mosaic arrangement each element of which acts at the right time and in the right way. Scattered observations suggest, however, that certain of the movements are correlative in nature—a result of interactions between or among various parts of the egg. The present paper is an attempt to establish the existence of such correlative movements and to ascertain their relationships to the autonomous movements.

2. The presumptive chordal region of the dorsal blastoporal lip has an autonomous capacity for extension (self-stretching) under diverse experimental conditions: various types of explantation, transplantation, and isolation *in situ*. It does not, however, possess an autonomous (inherent) capacity for invagination. This movement requires continuity between the presumptive chorda and the lateral portions of the marginal zone (*Randzone*) and is therefore correlative in nature.

3. The circumblastoporal region of the dorsal lip (presumptive pharyngeal entoderm and head mesoderm) has an inherent capacity for invagination which it expresses under diverse experimental conditions: various types of explantation, transplantation, and isolation *in situ*.

4. The lateral portions of the marginal zone, including the presumptive somites, lateral plate mesoderm, and tail mesoderm, have inherent capacities for stretching, involution, and invagination. But they are incapable of dorsalward convergence and of constriction over the yolk mass. These are correlative movements, for they take place only if there is continuity between the lateral marginal zones and the dorsal lip materials.

5. The capacity to exert these auxiliary effects is not limited to specific regions of the marginal zone. Thus the ventral marginal zone of the early gastrula can perform the gastrulation-promoting functions of the dorsal or dorsolateral marginal zones if placed in their positions.

6. The present experiments suggest that the correlative movements of gastrulation occur under the influence of forces exerted by the autonomous movements:

a) The presumptive chorda is invaginated by the inwardly directed tension or pull exerted upon it by the invagination and involution of the lateral marginal zones.

b) The lateral marginal zones are then pulled dorsalward and inward in the dorsal position by the autonomous stretching and simultaneous narrowing of the presumptive chorda.

c) The constriction of the blastoporal lips over the yolk mass is effected by the progressive withdrawal of marginal zone material by dorsalward convergence (a purse-string effect).

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PLATE 1

Fig. 1. Sagittal section through an explant composed of the dorsal blastoporal lip, sub-blastoporal presumptive entoderm, and much presumptive ectoderm. Specimen shown *in toto* in text figure 2, C.

Fig. 2. Transverse section through a ring embryo produced by implanting strips of presumptive ectoderm into slits cut through the marginal zone on both sides of the dorsal lip. A similar specimen is shown *in toto* in text figure 3, C.

Fig. 3. Sagittal section through a ring embryo like the one described in figure 2 of this plate.

Fig. 4. Transverse section through an explant composed of the dorsal lip, adjacent portions of the marginal zone, and presumptive ectoderm. Specimen shown *in toto* in text figure 8, D.

Fig. 5. Transverse section through an embryo which received two dorsal-lip implants in the ventrolateral marginal zones. See text figure 7, D and E.

EXPLANATION OF ABBREVIATIONS USED

Arch., archenteric cavity; *ch.*, primary chorda; *ch.¹* and *ch.²*, secondary chordas; *bp.*, blastoporal pocket; *ec.*, presumptive ectoderm; *en.*, presumptive entoderm; *hg.*, presumptive hind gut entoderm; *hm.*, presumptive head mesoderm; *m.*, mesoderm, *nt.*, neural tissue; *nt.¹* and *nt.²*, primary and secondary neural plates; *pr.*, presumptive chordal projection; *so. m.*, presumptive somite mesoderm; *tm.*, presumptive tail mesoderm.

DIFFERENTIATION AND GROWTH OF GASTRULAR ANLAGEN IMPLANTED HOMOPLASTICALLY INTO TADPOLES OF HYLA REGILLA

BY

MORGAN HARRIS

CONTENTS

DIFFERENTIATION AND GROWTH OF GASTRULAR ANLAGEN IMPLANTED HOMOPLASTICALLY INTO TADPOLES OF HYLA REGILLA

BY

MORGAN HARRIS

INTRODUCTION

TRANSPLANTATION of embryonic primordia into older hosts has been used extensively for studies on self-differentiation of anlagen from amphibian blastulae and gastrulae. Dürken (1925, 1926) devised the method of interplantation whereby grafts may be cultivated in the optic cavity of well-differentiated larvae after removal of the eye. The interplanted fragments develop at first in isolation within the optic cavity, later coming into association with the adjacent host tissues. In his experiments Dürken selected blastulae and gastrulae of *Rana temporaria* for donors; *Rana* and *Triton* larvae served as hosts. An indefinite area was removed from the animal hemisphere of the donor embryo, excluding so far as possible the mesodermal regions. Typical differentiation occurred in a large number of grafts. Notochord, cartilage, bone, muscle, nervous tissue, glands, and epithelium of several types were formed. As the tissues of the host surrounding the transplant were considered neutral in a morphogenetic sense, these results were taken as indicating self-differentiation of the ectodermal cells near the animal pole of the blastula or gastrula. This interpretation was not conclusive since the exact origin of the graft in the donor embryo could not be determined with certainty.

In 1929 Kusche extended these experiments to blastulae and gastrulae of *Triton* and *Amblystoma*, using an improved technique by which circumscribed primordia of known origin could be removed from the donor embryo. Presumptive medullary plate from the gastrula of *Triton* or *Amblystoma* in the optic cavity of older larvae gave rise to notochord, musculature, mesenchyme, occasionally to skin, and rarely to nervous tissue. A few entodermal grafts differentiated into notochord, but presumptive mesoderm formed only normal mesodermal derivatives.

Similar results were reported by Bautzmann (1929*b*), who excised pieces of blastulae or gastrulae of *Triton taeniatus* 150°–180° dorsal to the blastopore and transplanted these blocks of cells to the optic cavities of year-old *Triton* larvae. Chorda and muscle differentiated in the grafts. Whereas Kusche merely reported experimental results, Bautzmann came to important theoretical conclusions on the basis of his work. Considering the optic cavity to be a neutral environment, in contrast to his earlier views (1929*a*), he described the process of differentiation occurring in his grafts as "bedeutungs-fremde" development. By this term is implied differentiation of isolated anlagen, in the absence of organizing influences, into structures not ordinarily

formed by the cells in normal development. Accordingly Bautzmann assumed presumptive ectoderm to be determined in a labile manner not only for epidermis, but also for neural tube, notochord, and muscle. From this point of view development represents a gradual restriction of potencies until definitive structures are produced, in contrast to the principle of progressive determination from an indifferent state, as maintained by Spemann, Holtfreter, and others.

Holtfreter (1925, 1929a, 1929b, 1931a) developed techniques for culturing fragments from the gastrula in the coelom or lymph spaces of tadpoles. His first results (1929a), based on a study of both anuran and urodele types, indicated simple self-differentiation of mesoderm, entoderm, presumptive epidermis, and presumptive medullary plate. Later (1931a) he modified these conclusions as related to the development of presumptive epidermis and presumptive medullary plate in the coelom. About half of the transplants of presumptive epidermis in this location formed skin, but in the remaining number of grafts typical nervous tissue was formed. Likewise, presumptive medullary plate formed nervous tissue or skin with nearly equal frequency. In explaining these discrepancies Holtfreter assumed the existence of inductive factors, unknown in nature, in the body fluids of the host. With the foregoing exceptions, however, "bedeutungsfremde" development, as observed by Dürken, Kusche, and Bautzmann in the optic cavity, did not occur in grafts transplanted into the coelom or lymph spaces; notochord and muscle, for example, were not formed from ectodermal or entodermal regions.

Recently Emerson (1940, 1941) has pointed out the advantages of the regenerating tail blastema of anuran larvae as a medium for the growth of embryonic anlagen. Blastema mesenchyme proved to be a highly satisfactory environment for the morphogenesis as well as the histogenesis of embryonic organs. In his earlier paper Emerson described development of various regions of the embryonic brain, transplanted in an effort to bring about inductions in the blastema mesenchyme. No evidence was found to support the conclusions of Schotté (1937) that a lens may be formed from blastema cells under influence of the embryonic eye cup. Structures similar to ear vesicles, however, appeared to be formed by an inductive process in the blastema mesenchyme adjacent to grafts of embryonic medulla. In 1941 Emerson reported the results of transplanting gastrula ectoderm into blastema tissue. Typical epidermis, nervous tissue, suckers, cartilage, and dermis were formed. In 3 out of 169 grafts notochord and muscle appeared; these were believed due to accidental inclusion of mesodermal anlagen in the transplants. The discussion of these findings states: "The variety of ectodermal derivatives in the blastema, therefore, may be partially explained by favorability of the new location, combined with the release of the graft from its normal environment. These considerations obviously provide an explanation of the results only in a very limited and unsatisfactory sense."

Clear differences appear when the differentiation of presumptive ectoderm in larval hosts is compared to parallel explantation experiments in neutral salt solutions. Holtfreter (1931b, 1938a, 1938b) found that urodele ectoderm from

blastulae or gastrulae formed only an atypical epidermis *in vitro;* anuran ectoderm of similar origin formed larval suckers in addition. Alternatively, this difference is interpreted as being due to a special kind of development within the cells of the graft (Dürken, Bautzmann, Emerson) or to inductive factors in the body fluids of the host (Holtfreter, Spemann, 1938). This conflict may be due in part to the specialized character of individual experiments. To date, no comparative study has been made on the development of embryonic anlagen in different parts of the larval host to learn the relative importance of local influences together with the larval environment as a whole in determining the differentiation of the grafts.

The importance of a more general understanding of the factors governing development of embryonic anlagen in older hosts extends beyond whatever light these experiments may shed on the regulatory potencies of embryonic cells. In previous investigations the focus of interest has centered on the development of the graft itself, and its usefulness overlooked as a means of testing for the presence or absence of inductive phenomena among the tissues of the host. Inductive factors in larval tissues, if present, might conceivably affect regeneration, or the maintenance of tissue organization, concerning which little is known. It is likewise of interest to study more closely the growth and behavior of embryonic cells placed in intimate association with larval tissues. Development of such age-incompatible grafts may be compared to the pathological processes of healing, regeneration, and tumor growth, in which the active cells display characteristics seen normally only in embryonic stages. The present experiments, in which gastrular anlagen were implanted homoplastically into tadpoles, were undertaken with the foregoing general questions in mind.

I take this opportunity to acknowledge the generous assistance of Professor J. Frank Daniel, who by helpful criticism and suggestions has aided materially in the experimental work and the preparation of this paper.

MATERIALS AND METHODS

Eggs and tadpoles of the Pacific tree frog, *Hyla regilla,* provided material for the present investigation. Beginning gastrulae served as donor embryos in all experiments. Tadpoles to be used as hosts were either collected at the proper stage from ponds near Berkeley or raised from eggs in the laboratory. For the majority of experiments the tadpoles used were approximately 25 mm. in body length, with hind limbs represented by a short, straight outgrowth, without digits or joints. Operations were performed under a dissecting binocular microscope according to the usual microsurgical procedures. A 0.001-per cent solution of MS 222 in pond water was employed to anesthetize the tadpoles. All operations were performed under water; the tadpoles were held in place by celluloid bridges. Aseptic technique was found to be unnecessary because of the very high resistance of the larvae to infection. Operative mortality in all types of transplantation experiments here described was negligible.

Experimental and control animals were cultured in finger bowls containing pond water, and were fed daily with strained spinach. Bouin's fluid was used

to fix tadpoles for histological study. For dehydration and embedding the dioxane-paraffin method was used. Serial sections, cut at 8 μ, were stained with Harris's haematoxylin-eosin, iron haematoxylin–aniline blue (Koneff, 1936), and Mallory staining procedures.

EXPERIMENTAL WORK

GENERAL CONTENTS

The experimental studies to be described here fall into two major sections, dealing with transplantation of presumptive notochord and presumptive epidermis respectively. The choice of these gastrular rudiments for grafting was based on their contrasting embryologic characteristics. Presumptive notochord is strongly self-differentiating and its development within the larva serves as a control on whether the physiological characteristics such as oxygen tension and osmotic pressure within the larval body are suitable for differentiation. Implantation of presumptive notochord also gives a means for testing the ability of the embryonic organizer to affect tissues of postembryonic age. On the other hand presumptive epidermis in the gastrula is minimally determined, has relatively slight powers of self-differentiation, and, depending on the action of external stimuli, may develop into a variety of ectodermal and mesodermal derivatives. The course of development in grafts of presumptive epidermis which are implanted into tadpoles may reveal the presence or absence of inductive factors in larval tissues.

Grafts of presumptive notochord and presumptive epidermis were each implanted in several places within the host tadpoles. This variation in the site of transplantation was planned to give a series of relationships with different degrees of association between graft and tissues of the host. Grafts were placed in the empty optic cavity, loose connective tissue of the dorsal lymph spaces, cavity of the brain, and the muscles of the tail. Each series included a group of control animals which were operated upon without inserting a graft. Consideration was restricted to developmental aspects of the relation between graft and host. The question of pathological reactions by the larval body to implanted embryonic rudiments has been discussed previously (Harris, 1941).

TRANSPLANTATION OF PRESUMPTIVE NOTOCHORD

In this group of experiments, a rectangular block of cells was excised from the region of presumptive notochord, which in the beginning gastrula occupies a crescentic area slightly dorsal to the blastopore. The location of this rudiment was determined by vital-staining methods and a close correspondence was found with Vogt's map (1929). The region cut out for transplantation is indicated in figure 1* by dotted lines. So far as possible, only presumptive notochord was included in the grafts.

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* In this paper all illustrations are numbered in one series, 1–21. Figures 1 and 10 are in the text; all others are parts of plates 2–7.

DIFFERENTIATION IN THE OPTIC CAVITY

The operative technique differed somewhat from that employed by previous workers and will therefore be described in detail. The tadpoles to be used as hosts were anesthetized, and placed in a depression in the floor of the operating dish. The animals were oriented in such a manner that the dorsolateral surface on the right side was uppermost. An incision was next made through the dorsal rim of the conjunctival epithelium, extending anteroposteriorly for a distance equal to the diameter of the eye. Light pressure with forceps and probe was sufficient to force the eye out through this opening. Muscles, blood vessels, and nerves to the eye were severed by grasping these attachments with forceps as far proximally as possible on the median side of the eye and removing the eye with a probe. By exerting sufficient pressure with the forceps the cut end of the optic artery was usually clamped shut and bleeding thus prevented. Insertion of the graft was effected by means of microknife and probe. Considerable difficulty was experienced in placing the graft deep in the cavity of the eye by this means since the fluid in the empty eye socket apparently contains a small quantity of coagulum, which tends to tear grafts inserted too deep.

Healing of the incision made to remove the eye was rapid; the cut closed within a few hours and the processes of repair were completed in two or three days. The conjunctival epithelium provided a transparent cover to the culture chamber thus created, through which observations could readily be made on subsequent days. During the first three or four days after operation no visible changes were apparent in the graft other than a rounding in shape and an apparent slight increase in volume of the implant. An acceleration of growth then usually ensued, with the formation of irregular rounded protuberances on all sides of the graft. Through a continuation of these processes the structures developed from the transplant filled the optic cavity at the end of the third week and in some tadpoles bulged outward as blunt projections.

The period of most rapid growth occurred in the first month after operation. In the specimens kept beyond this time a gradual tapering off in growth rate was observed. In a few tadpoles, however, the grafts continued growth for some time and attained a size two or three times that of the host eye, projecting prominently from the head.

The processes of differentiation and growth in grafts of presumptive notochord to the optic cavity were further observed in histological examination of experimental animals. Sixty-two specimens in this series were preserved.
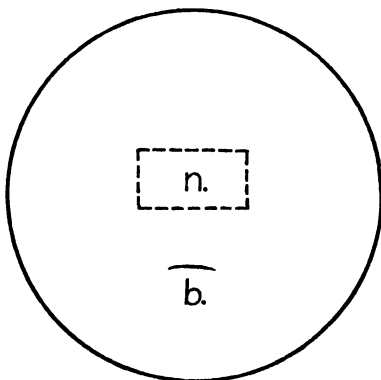

Fig. 1. Dorsal aspect of anuran gastrula. *b.*, blastopore; *n.*, presumptive notochord.

Of this number 19 were fixed at regular intervals of from 1 to 21 days after operation for the purpose of establishing continuity in the early phases of differentiation. The remaining specimens were cultured beyond the period of differentiation of the grafts, with postoperative age varying from 14 to 70 days.

The sequence of events following operation may best be described in a general discussion illustrated by specific experimental animals. Differentiation was first evident in grafts from animals fixed 2 days after operation. Notochordal tissue could be recognized in various regions of the transplant at this time. The notochord was formed in part by a localized vacuolization of cells, producing a weblike structure in the meshes of which yolk platelets and flattened nuclei bearing prominent nucleoli were present. Elsewhere in the graft, the cells retained an undifferentiated, yolk-laden appearance, except at the fringes, where occasional cells bearing yolk platelets differentiated into mesenchyme. Connective tissue from the host in many specimens grew in on the dorsomedian side of the graft and established contact with the transplant at this time. In other tadpoles the graft remained essentially free in the optic cavity.

During the third day after operation the development of notochord became more marked, and indications of nervous tissue appeared, but were confined for the most part to nuclear changes in portions of the graft where the nuclei appeared large and rounded and possessed the characteristic "checkerboard" distribution of chromatin granules. These cells formed solid masses or became arranged into irregular tubelike structures, the lumina for which appeared by separation of cells in the interior of the transplant. In one specimen muscle fibers were observed, the cross striations of which had not yet formed.

The following experiment illustrates the state of differentiation and relations of graft and host at the end of the fourth day (see figs. 2 and 3, pl. 2).

The symbols used in protocol numbers to designate individual specimens are as follows. The first letter refers to the site of implantation: O, optic cavity; S, dorsal lymph space; B, cavity of the brain; M, muscles of tail. The second letter indicates the gastrular region selected for transplantation: C, presumptive notochord; E, presumptive epidermis. Immediately following these letters is the serial number of the specimen, and, after a dash, the number of the slide to which reference is made.

OC 30–42. Postoperative age: 4 days.—The tissues of the graft form an irregular spherical mass in the optic cavity, in contact dorsally and medially with the adjacent connective tissue of the host (*cth.*, figs. 2 and 3, pl. 2). Among the components of the transplant are visible nervous tissue (*ns.*), notochord (*ch.*), muscle (*m.*, fig. 3). In all these tissues yolk platelets may be clearly identified, indicating that the tissues have a definite origin in cells of the graft. Three masses of notochord may be observed under higher magnification. The interstices between pieces of notochord are filled for the most part by nervous tissue, in which white and gray matter are present. Differentiation in the muscle fibers has not progressed far; fibrillae are present, but cross

striations have not appeared and the nuclei retain a spherical, embryonic character. A marked diminution in the number of yolk platelets throughout the graft is evident, although a few undifferentiated cells in scattered parts of the graft still present a yolk-laden appearance. Comparison of this graft with sections through a normal embryo of four days reveals a slight lag in differentiation in the transplanted piece, similar to that occurring in the majority of embryonic transplantation experiments. Both the graft and the tissues of the host in the neighborhood of the transplant appear uniformly normal. Leucocytes and free erythrocytes have disappeared from the optic cavity, indicating completion of the healing process after operation. An indentation (*in.*, fig. 2, pl. 2) in the conjunctival epithelium remains at the

TABLE 1

DIFFERENTIATION OF PRESUMPTIVE NOTOCHORD IN THE OPTIC CAVITY

Number of operations	48	Cartilage	17
Grafts resorbed	10	Skin	8
Nervous tissue	37	Eyelike structures	3
Notochord	38	Atypical epithelial cysts	5
Muscle	9		

region of original incision. No sign of vascularization of the graft by blood vessels of the host is apparent as yet, although capillary buds are present in the adjacent connective tissue.

Differentiation in the cells of the graft appeared to be nearly complete by 5 or 6 days after operation. A period of vigorous growth followed, recognizable histologically by an abundance of mitotic figures. The rate of growth and ultimate size of grafts showed appreciable variation, even in different parts of a particular type of tissue within a given transplant. The cause of these differences is obscure, although variation in the degree of vascularization of grafts by the host appears significant. Blood vessels began to grow into the graft during the first week, and in the following week they established normal vascular relations with structures of the transplant.

The general character of a well-differentiated graft is shown by the following specimen, illustrated in figure 4, plate 2.

OC 46–212. Postoperative age: 21 days.—Structures of the graft form an irregular ball of tissue which fills the optic cavity and bulges out laterally. Notochord (*ch.*, fig. 4, pl. 2), nervous tissue (*ns.*), and cartilage (*ca.*) may be recognized in the transplant. Especially noteworthy is the chaotic arrangement of these tissues; formation of definite organs is entirely lacking. Under a higher magnification mitoses may be observed in numbers in parts of the nervous tissue. Elsewhere in similar situations in the graft mitotic figures are rare. The adjacent tissues of the host appear normal and unaffected by the presence of the graft, aside from a slight physical displacement due to growth of the transplanted cells.

The products of differentiation from transplants in animals which were maintained 7 days or longer are summarized in table 1.

The high percentage of grafts resorbed may be attributed in part to initial difficulties of the graft in acquiring adequate vascular connections with the host. In every specimen in which a graft persisted, notochord was formed. Two types of growth were distinguished in the notochordal tissue. Where connective tissue formed a sheath around notochordal cells, irregular rod-like or spherical masses were formed (fig. 2, pl. 2). In the absence of a sheath, disorganized or diffuse growth of notochord occurred. In the majority of examples of this type the notochordal tissue was surrounded by nervous tissue, into which the cells burrowed intimately. Figure 5, plate 3, indicates the nature of this relation between the two tissues of the graft; there is no notochordal sheath. Ingrowth of notochord apparently had no injurious effect on the medullary tissue. Mitoses were often numerous in the nerve cells adjacent to ingrowths of notochordal tissue, and no evidence of degeneration was seen.

Nervous tissue formed a large part of nearly every graft, assuming a variety of appearances from simple differentiation of white and gray matter to the formation of brainlike structures. Irregular lobular masses of white and gray matter were seen frequently, in diverse shapes, containing many slit-like lumina or large cavities. These showed an obvious resemblance to central nervous tissue, although identification of specific regions was for the most part lacking. Typical nervous structures were observed in a few specimens, and included formations which resembled in general features the medulla, midbrain, telencephalon, spinal cord, and, occasionally, choroid plexus and ganglia. Nervelike outgrowths were found frequently.

Eyelike structures were observed occasionally. In two animals these were solid and surrounded by a dense layer of pigment, inside which was a layer of rods and cones. A typical optic cup was formed in a third graft. No lenses were formed. Muscle differentiated in a small percentage of grafts, and only in small amounts or in traces. Somites were not formed. Orientation of muscle fibers was lacking, the fibers forming an irregular mass except near notochord or cartilage, where most fibers were parallel to the surface of these structures. Skin differentiated in eight grafts, appearing in the form of hollow vesicles or cysts. Typical epidermis and dermis, including epidermal and corial melanophores, were observed, with the epidermal surface oriented uniformly inward. Inside the cysts were irregular masses or granules of pigment.

Differentiation of the parts of the graft into these structures apparently occurred at random, without relation to the surrounding structures of the host. Nervous tissue and notochord, which formed the bulk of the grafts, were found with equal frequency in all regions of the transplant. The disorganized character of the grafts may be ascribed to the disruption, by transplantation, of the normal correlations which exist between parts in the whole embryo.

Stress should be laid on the essentially normal histological character of the tissues of the grafts. Abnormal structures were formed in but few grafts, occurring there in traces only. These formations consisted of epithelial cysts of indeterminate character, the walls of which ranged from a squamous epithelium, with nuclei closely resembling adjacent connective tissue, to cuboidal, low columnar, or irregular epithelia.

DIFFERENTIATION IN THE DORSAL LYMPH SPACES

A short transverse cut was made through the skin in the median dorsal region, at the level of the hindbrain. Posterior to the line of incision a conspicuous pocket exists between skin and underlying muscle and neural structures, where the skin of the head rises to form the anterior end of the dorsal fin. With probe and operating knife the loose connective tissue was pushed toward either side in this cavity and the graft inserted. Subsequent contraction of the skin at the cut edges served to hold the transplant in place. Where the incision did not extend far laterally, bleeding from cut blood vessels was slight. Postoperative infection rarely occurred. Healing was rapid and fairly complete within a few days.

Histological examination was made of 36 specimens, including a chronological series fixed at intervals of from 1 to 3 weeks. Postoperative age extended up to 53 days. Differentiation in these experiments was essentially similar to that described for the previous series, except that the graft developed within the connective tissue of the host rather than in a free state. There was a mild leucocytic infiltration of the connective tissue near the operated region, but it disappeared in a few days as healing progressed. Loose erythrocytes from blood vessels ruptured during operation likewise gradually disappeared, and within a week the tissues in the region of the graft appeared uniformly normal. Yolk platelets were observed in differentiating notochord, nervous tissue, muscle, connective tissue, and skin, thus demonstrating the origin of these tissues from the cells of the transplant.

The following experimental specimen and corresponding illustration (fig. 6, pl. 3) are typical of a well-differentiated graft in the dorsal lymph cavity.

SC 90–211. Postoperative age: 21 days.—The structures of the graft form a bulky mass filling the space between the skin and surface of the muscles. Within the graft two irregular pieces of notochord (*ch.*, fig. 6, pl. 3) may be observed, a large area of nervous tissue (*ns.*), a piece of cartilage (*ca.*) adjacent to the notochord, a small skinlike vesicle (*sk.*), and two atypical epithelial cysts (*cv.*). Differentiation here is again purely histological, with no organization of the components of the graft with respect to one another. The cells of the transplant present a fresh, healthy appearance. Mitoses are not visible in the section selected for illustration, though found in certain other parts of the graft.

Growth of the structures of the graft has resulted in a slight deformation of the muscles of the host (*mh.*) and an abnormally wide separation of the skin from underlying structures. Aside from these influences of a physical character, the tissues of the host in the neighborhood of the graft appear uniformly normal.

The products of differentiation in grafts from animals cultured seven days or longer after operation are summarized in table 2 (p. 50).

These results are nearly the same as those obtained by culture of presumptive notochord in the optic cavity. Notochord, neural tissue, and cartilage formed the bulk of the grafts; muscle and skin were observed in smaller

amount in a few animals. A variety of ectodermal structures appeared, including typical olfactory epithelium in two grafts. In another animal a cyst composed of squamous epithelium and containing a placode of sensory epithelium was formed, resembling the *crista acoustica* of the ear.

Projections above the graft were seen in microscopic examination to be rodlike extensions of notochord. Mitoses were numerous at the distal tips of these projections. That the surrounding skin of the host had grown outward with the graft and was not merely passively stretched was shown by the presence of a large number of mitoses in the epidermis over the notochordal protuberance.

TABLE 2

DIFFERENTIATION OF PRESUMPTIVE NOTOCHORD IN THE
DORSAL LYMPH SPACES

Number of operations	29	Skin	7
Grafts resorbed	3	Olfactory epithelium	2
Notochord	22	Eyelike structures	3
Muscle	5	Sensory placodes	1
Nervous tissue	24	Atypical cysts	5
Cartilage	16		

Fusions of several types were observed between tissues of the host and those derived from the graft. In one, cartilage from the transplant continued smoothly without a line of separation into cartilage surrounding the neural tube of the host. Three skin cysts originating from the graft opened to the exterior through the epidermis of the host, so that the skin of host and graft formed a continuous layer. A third type of fusion was observed in one animal in which a tubelike structure of olfactory epithelium had developed an opening resembling an external naris through the skin of the host. Such a fusion seemed to occur most frequently where the skin had been incised.

DIFFERENTIATION IN THE CAVITY OF THE BRAIN

A longitudinal incision was made in the sagittal line through the skin and dorsal wall of the forebrain and midbrain. The cut edges were spread apart rapidly and the graft inserted with microknife and probe. The transplanted piece in the majority of specimens came to lie inside the cavity of the diencephalon or mesencephalon, in contact with the cut edges of brain tissue. Speed of insertion was essential; otherwise abundant bleeding and strands of fibrin formed by clotting rendered insertion of the graft difficult.

The operation caused severe shock in experimental animals, but recovery was uniformly successful if the preoperative anesthesia was not too deep and the medulla was uninjured during operation. A few specimens died within 24 hours after operation, either from infection or from undetected injury to vital centers in the medulla. Generally, however, healing proceeded satisfactorily, extending over a period of a week after operation. A few experimental animals exhibited deficiencies in motor coördination from the effects of the operation, but the rest were normal in external appearance and activity.

The internal development of the graft precluded possibility of gross observation; no external sign of the graft was visible until 2 or 3 weeks after operation, when about a third of the animals showed protuberances above the brain in the region of the graft, similar to those observed in transplants in the dorsal lymph spaces.

Fifty-seven experimental animals in this series were examined histologically. Of this group, 16 were preserved at intervals of from 1 to 21 days after operation. The remainder were maintained long enough to insure complete differentiation of the graft, that is, from 7 to 90 days.

The presence of yolk platelets in the graft was particularly advantageous histologically in distinguishing the cells of the graft and host. Nervous tissue

TABLE 3
Differentiation of Presumptive Notochord in the Brain

Number of operations	45	Muscle	13
Grafts resorbed	11	Cartilage	16
Nervous tissue	?	Skin	5
Notochord	22	Atypical epithelial cysts	7

formed from various regions of the graft, often in direct contact with the cut edge of the brain, where fusion with the nervous tissue of the host occurred. The transplant usually became attached to the roof of the diencephalon or mesencephalon, hanging down into the cavity of the brain or projecting outward.

The typical features of a well-differentiated graft in the brain are illustrated by the following specimen, shown in figure 7, plate 3.

BC 87–352. Postoperative age: 35 days.—A large mass of notochord (*ch.*) forms the bulk of the graft, which hangs down into the cavity of the diencephalon as an extensive irregular mass. Nervous tissue (*ns.*) is also found in the graft. Cartilage and nasal epithelium are to be observed in other sections. A smooth fusion is found between the graft and roof of the brain on each side, while secondary fusions are developing between graft and floor of the brain. Under higher magnification a region of muscle (*m.*) may be observed dorsally, representing part of a layer surrounding a projection of notochord which may be seen a few sections posteriorly.

Table 3 presents in condensed form the results of differentiation in this series for all experimental animals maintained seven days or longer.

More grafts were resorbed in this series of experiments than in grafts to the optic cavity or dorsal lymph spaces, possibly because of difficulty in obtaining proper vascularization in the brain. It was not possible to estimate the incidence of nervous tissue in this series, for the fusion of nervous tissue between graft and host was intimate and complete. A line of separation was visible only up to 4 or 5 days after operation, when differentiation was visible but yolk platelets still were present in the implant. At this stage nervous tissue, derived from the transplanted cells, could be demonstrated in all grafts. Later in the postoperative period the distinction between nervous tissue of host and graft was lost. In three animals a prominent mass of nervous

tissue unaccompanied by other structures was found in the roof of the diencephalon or mesencephalon. A specimen of this type is shown in figure 8, plate 4. No such structures were observed in control animals, in which the operation had been performed without insertion of a graft. The nervous tissue formed was atypical for the region in which it was found, containing irregular lumina and abnormal distribution of white and gray matter. Notochord was found in small amount in the middle of one mass of nervous tissue. Thus three transplants apparently differentiated primarily into nervous tissue, which fused intimately with the surrounding walls of the brain.

Diffuse growth of notochord, when the cells were not surrounded by connective tissue as mentioned previously, was observed in several grafts. One of these is shown in figure 9, plate 4. The transplant here appears to have differentiated completely into notochordal cells (*ch.*), which in the absence of a sheath of connective tissue have burrowed intimately into the surrounding brain tissue (*ns.*). The growth pressure of the developing graft has caused a considerable diminution or dislocation in the amount of brain tissue normally found at this level, although the animals in life appeared normal in locomotor activity.

DIFFERENTIATION WITHIN MUSCLES OF THE TAIL

A longitudinal incision was made with microknife and probe through the skin of the tail, parallel to and slightly above the muscles, and extending from the base of the tail posteriorly for one-third of its length. A pair of jeweler's forceps, the terminal portions of which had been sharpened to simulate a pair of surgical scissors, was inserted into the dorsal surface of the muscles on one side and pressed together, producing a clean longitudinal incision into the muscles without the excessive bleeding which followed use of iridectomy scissors. The graft was inserted with microknife and probe, and came to lie in direct contact with the cut surfaces of the muscle. Healing was rapid, becoming complete within a few days.

The graft was not visible after operation and gave no external sign of its presence for a week or two, after which a lateral bulge of the muscles surrounding the graft was visible in many specimens and gradually became larger with increasing age. Simultaneously, protuberances were observed at the dorsal surface of the original incision into the muscle. These knobby structures frequently extended into the lymph spaces above the muscle, forming irregular masses. Projections lateral from the tail, similar to the finger-shaped or pointed projections above grafts in the dorsal lymph spaces or brain, were not found here.

A total of 48 animals was preserved for histological study, including specimens fixed in the early stages of differentiation. Postoperative age in these experiments ranged up to 90 days.

Microscopic examination revealed that damage to the tissue of the host was more extensive in this operation than in those previously described, and resulted in extensive processes of repair and regeneration in the neighborhood of the graft. Leucocytic invasion of the region occurred during the first few

days after operation. Four or five days after operation, regenerating muscle cells began to accumulate; the cells were abundant near the graft a week after operation and superficially were similar to young notochordal cells of the graft, from which they differed slightly in nuclear appearance. The injured muscles regenerated within a period of two to three weeks.

Differentiation of grafts within muscles of the tail followed closely the pattern discussed above in transplants of presumptive notochord to other regions of the body. Table 4 presents the results of differentiation in grafts placed within muscles of the tail, where the hosts were maintained for seven days or longer after operation.

The small number of grafts resorbed in this region seems to reflect the favorable conditions for vascularization of the graft by adjacent vessels of the host.

TABLE 4

DIFFERENTIATION OF PRESUMPTIVE NOTOCHORD WITHIN MUSCLES
OF THE TAIL

Number of operations	36	Nervous tissue	31
Grafts resorbed	5	Cartilage	18
Notochord	26	Skin	5
Muscle	1	Atypical cysts	9

The products found in these grafts are essentially the same as those obtained from differentiation of presumptive notochord in the optic cavity and dorsal lymph spaces. No data on the occurrence of muscle in these grafts were available here, since muscle of graft and host could not be distinguished after the period of differentiation. That presumptive notochord can form muscle in this region, however, was demonstrated in at least one graft, where yolk platelets were observed in developing muscle fibers.

Growth of notochord in the tail followed a much more typical course than in the locations previously discussed. Rodlike structures, indistinguishable in general from normal notochord, were formed in about half the grafts and were oriented uniformly with the long axis in an anteroposterior direction. Fusions between nervous tissue of the graft and spinal cord of the host were observed in several animals. In two specimens the skin of the graft was connected with the skin of the host. The muscles of the host appeared to be unaffected by the presence of the graft, except for a physical displacement. The embryonic tissue did not influence regeneration of the tail muscles.

TRANSPLANTATION OF PRESUMPTIVE EPIDERMIS

To insure validity of the results obtained from transplanting presumptive epidermis it was deemed essential to avoid inclusion of mesodermal anlagen in the grafts. A careful check was therefore made by vital-staining methods of the normal fate of the region to be grafted. The center of the block of cells cut out for transplantation was situated on a meridian directly opposite the blastopore. The bottom edge of this piece was slightly above the yolk mass, and

the block extended dorsally to a line about 30° below the animal pole. The lateral dimension of this piece was somewhat greater than this dorsoventral extension. All stains placed on this area in early gastrulae were later observed in the skin of older embryos which developed from these eggs. Figure 10* indicates diagrammatically the region removed for transplantation.

DIFFERENTIATION IN THE OPTIC CAVITY

To avoid injury to the thin block of presumptive epidermal cells, the grafts were placed in a peripheral position in the optic cavity, directly under the conjunctival epithelium. A deeper implantation by the technique used proved unsatisfactory, since the graft was then torn by strands of fibrin formed in the cavity after operation. The initial position of the graft is of significance in interpreting results.

Fig. 10. Lateral aspect of anuran gastrula. *b.*, blastopore; *e.*, presumptive epidermis.

Gross observation of the transplants in the postoperative period revealed fewer changes than were observed in grafts of presumptive notochord in the same location, and growth was much slower and far less extensive, and irregular masses of tissue did not fill the optic cavity by rapid growth. Instead, grafts increased in volume only gradually, without appreciable change in the ovoid or spherical shape assumed soon after operation. After about three weeks the grafts appeared as translucent vesicles, in the walls of which melanophores were visible. The size eventually attained by these structures was uniformly small, the transplant occupying only a part of the optic cavity.

Fifty-four specimens were preserved for histological study. To insure complete differentiation, 31 of these were maintained for 3 weeks or longer. The remainder were fixed at earlier stages to provide information on the intermediate processes of differentiation. Microscopic examination showed that the grafts remained free in the optic cavity for about two days, after which connective tissue from the adjacent regions of the host grew in and surrounded the transplants. All grafts retained their peripheral position in the optic cavity, and apparently made no contact with muscles or cut nerves at the base of the cavity.

All grafts differentiated into epidermis. The beginning stages of this process, in which pigment formed and vacuolization took place, were comparable in nature to similar processes occurring in the normal embryonic skin. At first these phenomena were found irregularly throughout the graft, but gradually became confined to cells in the interior as connective tissue from the host surrounded the transplant. The layer of cells situated peripherally with refer-

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* In this paper all illustrations are numbered in one series, 1–21. Figures 1 and 10 are in the text; all others are parts of plates 2–7.

ence to the graft, and in direct contact with connective tissue, differentiated after five or six days into rectangular unpigmented cells similar to those of the basal layer in normal skin. A conspicuous basement membrane subsequently appeared around the graft.

Internally, the cells of the graft appeared unpolarized, and irregular spaces, appearing at random through coalescence of vacuoles, contained masses of pigment and disintegrating yolk platelets. A heavy accumulation of pigment was apparent in cells directly bordering the cavities. These cavities gradually merged, finally forming a single central lumen surrounded by thick walls, much pigmented on their inner surfaces. Further differentiation resulted in thinning of the walls to the width of normal epidermis and the appearance of epidermal melanophores. These processes lasted approximately two to three weeks.

TABLE 5

DIFFERENTIATION OF PRESUMPTIVE EPIDERMIS IN THE OPTIC CAVITY

Number of operations	39	Atypical epithelial cysts	2
Grafts resorbed	8	Other structures	0
Epidermis	31		

A typical dermis, including dermal melanophores, was also formed, apparently from cells of the host. No evidence of differentiation of the cells of the graft into connective tissue or other dermal components was found. These points may be illustrated by the following example (fig. 11, pl. 4; fig. 12, pl. 5).

OE 51–191. Postoperative age: 19 days.—The graft consists of a vesicular or cystlike formation of skin (*sk.*, fig. 11, pl. 4), lying in contact with the conjunctival epithelium (*cj.*, fig. 11, pl. 4; fig. 12, pl. 5) and surrounded by the connective tissue (*ct.*, fig. 11, pl. 4) of the host. The outer margin of the epidermis (*ep.*, fig. 12, pl. 5) of the graft, considered morphologically, is oriented toward the interior of the cyst. The arrangement of cells in the layers of the epidermis is fairly normal, though vacuoles are still present in some of the cells and conspicuous solid balls of pigment (*pg.*, fig. 12, pl. 5) may be seen. External to the epidermis is a definite basement membrane (*bm.*, fig. 12), outside which is a slight accumulation of connective tissue. The interior of the cyst contains irregular masses of pigment and the products of disintegration of yolk platelets. All cells of the graft and neighboring connective tissue appear healthy and normal.

The results show the consistent formation of epidermis from presumptive epidermis in this location. With the exception of two tiny epithelial cysts of indefinite nature, no other structures were formed. The transplants invariably formed a single vesicle, situated either free within the optic cavity except for the surrounding connective tissue (17 examples), or in contact with the dermal layer developing beneath the conjunctival epithelium (14). Fusion between the skin of the graft and conjunctival epithelium occurred in 4 animals, giving rise to an opening from the cavity of the cyst to the exterior.

DIFFERENTIATION IN THE DORSAL LYMPH SPACES

The technique used in transplanting into the dorsal lymph spaces was followed closely in this series of experiments. The gross changes in the graft during the postoperative period paralleled those described for differentiation of presumptive epidermis in the optic cavity. Extensive growth did not occur in any of these specimens. After three weeks of culture the grafts typically had formed one or more small clear vesicles, usually somewhat flattened. Melanophores eventually appeared in the walls of these structures.

Histological examination was made of 32 specimens, including 28 which had developed for 3 weeks or more. The results are indicated in table 6.

TABLE 6

DIFFERENTIATION OF PRESUMPTIVE EPIDERMIS IN THE
DORSAL LYMPH SPACES

Number of operations	28	Cartilage	3
Grafts resorbed	4	Traces of nervous tissue	2?
Skin	23	Traces of olfactory epithelium	1?
Horny "teeth"	4	Atypical epithelial cysts	10
Sensory placodes	14		

Skin was found in the majority of transplants, and formed the major part of the grafts. It appeared as cystlike structures, which were formed by processes similar to those observed in the optic cavity. The physical effects of surrounding connective tissue produced flattening of the cyst, often leading to a division of the central cavity into numerous small lumina. In some grafts solid platelike formations of skin were observed.

In addition to the formation of skin, over half the specimens exhibited thin-walled epithelial vesicles bearing sensory placodes. These placodes, often bearing sensory hairs, consisted of a basal layer of cells with oval nuclei vertically oriented, and sometimes a superficial layer of flattened cells. In a few grafts, tiny masses of cartilage were found adjacent to sensory placodes.

In figure 13, plate 5, a typical graft with the structures just described is shown. A brief summary of this specimen follows.

SE 53–362. Postoperative age: 36 days.—A vesicle of skin (*sk.*, fig. 13, pl. 5) containing a divided lumen is found in the loose connective tissue beneath the skin of the host. A conspicuous basal membrane (*bm.*) has been formed, internal to which is a fairly typical epidermis (*ep.*). Pigment is present in the walls and interior of this cyst. An irregular vesicle, composed of squamous epithelium and bearing a sensory placode (*sp.*), lies in contact with the skin from the graft.

Epithelium of the type found in the lips and anterior end of the buccal cavity was clearly recognizable in a few grafts, although it often graded indistinguishably into skin. Typically, the cells and nuclei in this epithelium were larger and more regular than those of epidermis. Other points of difference from skin were the growth of buccal epithelium predominantly in flat,

platelike structures rather than in the form of vesicles, the lesser amount of pigment in the cells, and the presence in nearly every transplant of this type of horny structures, similar to the "teeth" and "jaws" of normal tadpoles. These formations may be illustrated by the following specimen (fig. 14, pl. 5; fig. 15, pl. 6).

SE 58–352. Postoperative age: 35 days.—The entire graft has differentiated into buccal epithelium (*be.,* fig. 14, pl. 5; fig. 15, pl. 6). This appears as a flat platelike structure containing small lumina (*l.*) and is folded and fused together at various points. The large space visible in the center of the graft is not a true lumen, but is continuous posteriorly and anteriorly with the adjacent lymph spaces. In one of the minor spaces within the buccal epithelium there are horny structures (*h.,* fig. 15, pl. 6) similar to the components of the normal "teeth."

Atypical epithelial structures were found frequently, but they never formed an appreciable proportion of the structures of the grafts. These took the form of small irregular vesicles, lined by variable types of epithelium.

The structures previously described were frequently in loose contact with connective tissue covering the dorsal muscles. To determine whether the presence of muscle beneath the connective tissue might account for these structures, a few experiments were conducted in which grafts of presumptive epidermis were placed in the ventral lymph spaces posterior to the digestive tract and in front of the cloaca. Here no contact with muscles was possible. The resulting structures were substantially like those in the dorsal lymph spaces: skin, thin-walled vesicles with sensory placodes, buccal epithelium, and atypical epithelia.

Two other types of structure appeared in the dorsal lymph spaces and may be connected with the presence of muscle. In two grafts a small patch of tissue was formed, the nuclei of which closely resembled those found in the cells of the central nervous system. White and gray matter were not distinguishable. In both specimens this piece of tissue was in close contact with the thin layer of connective tissue directly covering the muscles. Traces of nasal epithelium were observed in a third graft, this structure likewise having formed adjacent to the muscles of the host.

DIFFERENTIATION IN THE CAVITY OF THE BRAIN

The technique used in transplanting presumptive notochord was followed here. During the entire postoperative period no sign of the graft was visible externally. The animals were normal in outward appearance and activity.

Forty-seven animals were examined histologically. Of these 26 were preserved 3 weeks after operation, 17 at seven days, and the remainder at earlier stages. In a number of specimens grafts were either attached to the roof of the brain or, occasionally, were free in the cavity of the brain.

In many animals the graft was attached by its dorsal margins to the roof of the brain and lay between the cut edges of nervous tissue. The dorsal surface of the graft was exposed to the loose connective tissue above the brain, whereas the major part of the transplant projected freely into the ventricle

below. Contact with nervous tissue was restricted to a narrow dorsolateral region on either side of the graft. Differentiation of the grafts developing in this position proved particularly instructive in interpreting the results obtained in this series. During the first week after operation, vacuolization and formation of pigment occurred in the transplant, and, on the side of the graft and in contact with connective tissue, were eventually confined to the cells in the interior of the transplant. The peripheral cells differentiated into a basal layer of typical epidermal cells, next to which a basal membrane was found. In the part of the graft which projected downward into the cavity of the brain, formation of pigment and vacuolization was irregular throughout

TABLE 7

DIFFERENTIATION OF PRESUMPTIVE EPIDERMIS IN THE CAVITY
OF THE BRAIN

Number of operations	26	Buccal epithelium	3?
Grafts resorbed	20	Horny "teeth"	3
Atypical epidermis	6	Traces of nervous tissue	2?

the graft, becoming especially prominent on the margins of the transplant. No polarization or differentiation occurred in this mass, the cells of which retained a yolk-laden, irregular appearance. Beginning at 4 or 5 days after operation, some pigmented cells separated from the edges of this region, and assumed a rounded shape. They contained a large amount of cytoplasm filled with yolk platelets, pigment, and vacuoles. A large unspecialized nucleus, spherical or ovoid in character, was found in the cells.

Numerous isolated cells of this character occasionally were clumped together in loose aggregates throughout the cavities of the diencephalon and mesencephalon. Although these cells looked abnormal cytologically, no degeneration in either cytoplasm or nucleus was evident for a week or more after separation. These loose cells disintegrated later.

Reference to the following experimental specimen (see figs. 16 and 17, pl. 6) will clarify this sequence of events.

BE 52–71. Postoperative age: 7 days.—The graft (*gr.*, fig. 16, pl. 6) is visible as a tonguelike mass in the roof of the brain. Cells of the transplant are in contact dorsally with the connective tissue of the host; here differentiation along an irregular boundary of a basal layer of epidermis (*ep.*, fig. 17, pl. 6) may be seen. Vacuolization and pigment are not present in this layer. The ventral part of the transplant, projecting freely into the ventricle of the brain, is unpolarized and shows separation (*s.*) of pigmented cells at the margins. Elsewhere in the cavity of the brain are loose cells (*l.*, fig. 16, pl. 6) derived by separation from the graft. In other sections these free cells are more prominent. The transplant is in contact laterally with the edges of the roof of the brain. The graft and tissue of the host are not smoothly fused here, although they are in intimate contact.

Differentiation in grafts after three weeks of culture in the brain is shown in table 7.

In most specimens a definite graft was not found in the brain at 3 weeks after operation. Numerous loose pigmented cells of the type previously described were in the cavity of the brain, and many small vesicles of skin were in the connective tissue directly over the roof of the brain, indicating that a continuation of the processes observed at earlier stages had resulted in a separation of the cells of the graft from one another, except where the transplants had come in contact with connective tissue. In a few animals a mass of epidermal cells, atypical in shape and general appearance, was suspended from the roof of the brain. Within this mass, buccal epithelium appeared in some specimens, together with the horny structures described previously. Buccal epithelium and horny structures were observed also in the interior of one graft which was completely free within the ventricle of the brain.

Indications of nervous tissue from the grafts appeared in two questionable grafts. In each a tiny mass of white and gray matter, to which a few loose pigmented cells were closely adherent, was found at the region of incision as an apparent part of the graft.

No sign of the formation of connective tissue by the cells of the graft was observed. In all specimens where a clear separation of graft from the connective tissue of the host was present, connective tissue could not be demonstrated in or around the graft.

DIFFERENTIATION WITHIN MUSCLES OF THE TAIL

Presumptive epidermis was transplanted into the tail by the methods described previously. During the second and third weeks after operation marked changes resulted in the region of the graft. In contrast to the effects of transplanting presumptive epidermis to other locations, heterogeneous structures were formed by the grafts, and were accompanied by vigorous growth. In many specimens these processes resulted in extension dorsally of the graft into the lymph spaces above the muscles, where globular opaque masses, translucent vesicles, and irregular platelike structures were observed. Frequently, internal growth of the transplant caused the appearance of a prominent lateral bulge in the muscles of the host. In general appearance these phenomena were unlike those observed in grafts of presumptive epidermis in other locations, and resembled more closely the sequence of growth processes previously noted in transplants of presumptive notochord into the tail.

For further investigation into the nature of these differentiations, 52 animals were preserved and sectioned for microscopic study. Thirty-one specimens were cultured for 3 weeks or longer after operation; the remaining animals were preserved at earlier stages. It will be advantageous to present first the results of complete differentiation in this series obtained from study of animals cultured 21 days or longer. Consideration will be given subsequently to the intermediate formative processes. Table 8 (p. 60) summarizes the products of differentiation found in grafts three weeks after operation.

The frequent occurrence of nervous tissue is most striking. The medullary formations assumed a variety of appearances, recognizable by typical white

and gray matter. Prominent vesicles of nervous tissue, brainlike in character, were formed in two grafts. A description follows (see fig. 18, pl. 7).

ME 57–212. Postoperative age: 21 days.—The graft consists of a large hollow mass of nervous tissue (*ns.*, fig. 18, pl. 7), oblong or oval in cross section. White and gray matter are clearly visible. Several large eccentrically placed lumina (*l.*) are present. Ventrally and medially the graft is in contact with muscle fibers of the host; on dorsal and lateral sides the graft has grown beyond the boundaries of the muscle into the lymph spaces. Also present in the graft, although not visible here, is a piece of typical choroid plexus.

TABLE 8

DIFFERENTIATION OF PRESUMPTIVE EPIDERMIS WITHIN MUSCLES
OF THE TAIL

Number of operations	31	Olfactory epithelium	6
Graft resorbed	5	Sensory placodes	13
Nervous tissue	23	Buccal epithelium	7
Notochord	2	Horny "teeth"	6
Cartilage	21	Skin	21
Muscle	?	Atypical epithelial cysts	11

In 8 specimens formations resembling spinal cord were noted. The degree of similarity was variable. In one, a highly typical neural tube, formed from the graft, lay among the dorsal muscles in the midline. In histological character, size, and orientation this structure was nearly indistinguishable from the normal neural tube of the host lying a short distance below. Other grafts exhibited tubelike formations, in which the gray matter was concentrated about the lumen or dispersed irregularly.

In many specimens, however, there were irregular lobular masses of white and gray matter with no assignable similarity to a particular part of the central nervous system. The amount of medullary tissue that was formed appeared to bear a direct relation to the degree of contact between the structures of the graft and the adjacent muscles of the host. Where the graft was predominantly surrounded by muscle fibers, the cells of the transplant differentiated almost uniformly into nervous tissue. An example of this type is shown in figure 19, plate 7, and is described below.

ME 54–212. Postoperative age: 21 days.—A flattened platelike mass of nervous tissue, nearly solid, comprises the major product of differentiation of the graft. White matter (*wm.*) and gray matter (*gm.*) are irregularly distributed within this structure. The transplant is in close contact with muscle fibers (*m.*) over a considerable area, and has grown somewhat laterally at one side and inward toward the notochord on the other. No other structures of the transplant are visible here. In other sections through the graft a small piece of cartilage may be noted.

Where the association between transplanted cells and muscles of the host was less intimate, nervous tissue formed in lesser amount, and the remainder of the graft differentiated into other structures.

Ganglia were observed in 8 grafts. These occurred most frequently in grafts with but a small amount of nervous tissue; a ganglion was the only nervous structure in one graft. The cells in these ganglia exhibited a typical cytological structure, with large nuclei containing prominent nucleoli. Aggregation of the cells into groups was irregular, however, and in general did not give rise to the characteristic structure of normal ganglia. In addition to these formations, isolated cells exactly like those found in ganglia were often observed among the muscle fibers adjacent to the graft.

A high percentage of grafts contained cartilage. Here again the formations varied appreciably. Five grafts contained large, irregularly lobulated masses of cartilage. In one the ventral part of the cartilaginous mass was surrounded by muscle fibers, whereas the dorsal side projected into the lymph spaces. In the other four the graft occupied a median position, with the cartilage near or in contact with the notochord of the host ventrally, and in close association laterally with muscle fibers. In most grafts there were small, multiple pieces of cartilage. Although these masses were often close to the notochord of the host, the cartilage in several had formed entirely within the muscles or in conjunction with other structures of the graft, well separated from the notochord. The occurrence of cartilage is demonstrated in figure 20, plate 6.

ME 49–213. Postoperative age: 21 days.—A rounded or oblong mass of cartilage (*ca.*) has developed out of the transplant, and lies partly within the muscles (*m.*) of the host and partly in the lymph space (*l.*). Elsewhere in the graft is a prominent mass of nervous tissue, not visible here.

Differentiation of cartilage occurred only where the tissues of the graft were in contact with, or in the vicinity of, axial structures of the host; it did not occur, for example, in the dorsal lymph spaces above the muscle.

A third type of differentiation was indicated by the appearance of notochord in two transplants. One of these, shown in figure 21, plate 7, may be described as follows.

ME 56–214. Postoperative age: 21 days.—The external characteristics of the graft during the period of differentiation are of importance in interpreting the final positional relations of the structures formed. The graft was placed completely within the muscles of the tail, but in the course of a week it commenced to grow dorsally into the lymph spaces. The structures of the graft extended rapidly into the lymph space, there forming a prominent anteriorly directed projection. A section through this anterior region of the graft (fig. 21, pl. 7) reveals the presence of notochord (*ch.*) within an open shell composed of buccal epithelium (*be.*). Nervous tissue and cartilage occur more posteriorly, where the graft is partly surrounded by muscles.

Olfactory epithelium was arranged in typical tubular form in several specimens, forming the major part of the graft in two. These nasal structures were developed in connective tissue between the dorsal muscles or in direct contact with muscle fibers. Whether muscle fibers had differentiated from the grafts in this group of experiments was uncertain, since the fibers of graft and host could not be distinguished after the period of differentiation. Further evidence on this question will be presented later.

In addition to these structures, differentiations similar to those occurring in the dorsal lymph spaces were noted; these included buccal epithelium, horny "teeth," epithelial vesicles with sensory placodes, skin, and atypical epithelial cysts. The structures showed a higher degree of development in the tail than in the dorsal lymph space. Buccal epithelium was extensively developed in one particular specimen. Here the arrangement of the epithelium was similar in shape to the outlines of the anterior end of the buccal cavity, but was oriented 90° from the normal position, in conformity with the available space for growth. Conical papillae, characteristic of the buccal cavity in this region, were found prominently in the structures of the graft, as were also the horny structures described previously.

Vesicles composed of squamous epithelium and possessing sensory placodes occurred in a large number of grafts, either in the lymph spaces above or, occasionally, in contact with muscle. The placodes were especially well developed and in many specimens bore prominent sensory hairs. The resemblance of these placodes to the *cristae acousticae* of the ear was usually apparent, although many placodes showed only a general histological similarity. Skin formed in nearly every graft, producing vesicles or cysts in the lymph spaces above the muscles; skin did not form in or close to muscle. This fact will be of importance in subsequent discussion.

Atypical epithelial structures occurred here more frequently and in greater variety than in grafts of presumptive epidermis elsewhere; all were vesicular, of varying size and shape, and were composed of squamous, cuboidal, or irregular types of epithelium. To determine whether these products of differentiation were derived from the cells of the transplant or from the surrounding tissues of the host, a study was made of grafts in earlier stages of differentiation. Careful examination was made of 21 animals at 7 days after operation. Recognizable tissues had developed by this time, but many cells in the graft still possessed yolk platelets as a proof of their origin from the transplanted presumptive epidermis. The presence of yolk platelets was established in developing nervous tissue in numerous grafts. Identity of the nervous structures was made through the presence of white and gray matter. Another specimen showed yolk platelets in the meshes of developing notochord, and in a third graft there was clear evidence of the formation of muscle: typical fibers exhibiting cross striations had formed, while a few yolk platelets were still visible within the fibers. Yolk platelets were not observed in developing cartilage, possibly because the cells had first to go through a mesenchymal stage in which the yolk platelets were lost. Cartilage was probably derived from the graft, however, since no other cartilage is normally present in this region of the host.

DISCUSSION

Transplantation of the presumptive notochord of the gastrula into the optic cavity, dorsal lymph spaces, brain cavity, and muscles of the tail demonstrates the ability of the notochord to differentiate successfully in a variety of environments within the larva. Since this development is similar in nature to that reported by Holtfreter (1938*a*, 1938*b*) for explants of presumptive noto-

chord in salt solution, it would appear that surrounding larval tissues do not affect the processes of self-differentiation within the implant. The great variety of structures developed in these transplants may be regarded as evidence of the manifold potencies residing in the notochordal anlage before gastrulation.

The importance of connective tissue in the growth of notochord has been shown in these experiments. Wherever notochord developed in the absence of connective tissue, diffuse or irregular growth occurred, either freely into a cavity or into nervous tissue. A typical notochordal sheath, rodlike in shape and growing by extension of the notochord, was attained only where connective tissue surrounded the notochordal cells. These facts substantiate similar conclusions reached by Holtfreter (1939) in isolation experiments with notochordal cells.

The projections noted above the graft in the dorsal lymph spaces and brain cavity may be regarded as an expression of the inherent tendency of notochord to elongate, discovered by Bautzmann (1928). The failure of notochord to grow outward in such projections from the tail may be due to the more favorable mechanical conditions for expansion in the axis of the tail.

The sequence of developmental processes observed in grafts of presumptive notochord may be resolved into three phases. In the first, differentiation took place, accompanied by utilization of yolk in the cells; growth was slight, and appeared to be unaffected by the environment. In the second, abnormally rapid growth occurred, as shown microscopically by the presence of great numbers of mitoses. In the final phase, growth of the graft tapered off to the level of the surrounding tissues.

The phenomena observed during the first two of these periods can be explained very well on the basis of a theory of regulatory growth advanced recently by Twitty (1940), together with the further assumption that until differentiation and resorption of yolk are effected the cells do not assimilate food materials from the surrounding medium. Evidence for this view is given by Fischer (1937), who found that entodermal cells of *Amblystoma* in plasma cultures did not appear to be influenced by the culture medium or to show noticeable growth until resorption of yolk was completed.

Recent measurements by Twitty and van Wagtendonk (1940) show a rise in the level of amino acids in the blood stream of larval *Amblystoma* with increasing age. It is probable that these data are applicable also to other amphibian types. Hence the period of abnormally rapid growth in the present experiments may, as suggested by Twitty, be explained by the high nutritive level of the host, and furthermore would appear to be a regulatory process leading to reduction of the age differential between graft and host. That this difference does disappear is indicated in metamorphosing specimens, where tissues of the graft showed certain metamorphic changes simultaneously with those of the host, even though transplantation had been performed only a month previously. Thus, the cartilage transformed into bone and glands formed in vesicles of skin in the grafts. These processes were the same as the normal changes occurring in the body of the host. A tapering off of this period of rapid growth in the transplant would thus seem to occur when the

physiological age of the graft became comparable with that of the surrounding tissues. It is uncertain whether the eventual decline of growth rate in the transplant is due to a progressive loss in assimilative power of the grafted cells with increasing age, as is suggested by Twitty on the basis of other experiments in regulative growth.

No evidence was found that the embryonic organizer, as represented in presumptive notochord, is capable of affecting tissues of postembryonic age. All disturbances noted in the tissues of the host consisted of purely physical displacements, resulting from the growth pressure of the developing graft. One possible exception occurred in specimens in which projections of notochord growing outward from the body caused the overlying skin to grow out simultaneously.

The essentially normal character of differentiation in grafts of presumptive notochord in all locations serves as a control for interpretation of the development of presumptive epidermis, similarly placed. Results of culture of presumptive epidermis in the optic cavity, where skin formed as the sole product of differentiation in all the grafts, suggest that, contrary to the views of Holtfreter (1931a) and Spemann (1938), the lymphatic fluids within the optic cavity are not inducing in nature. In addition, these results indicate that mesodermal anlagen were not included in the piece of presumptive epidermis selected for transplantation, thus providing a check on the results obtained from similar grafts in other locations. Presumptive epidermis in the dorsal lymph spaces differentiated into epidermis, horny "teeth," sensory placodes, and numerous atypical epithelial cysts. The appearance here of other derivatives in addition to epidermis may be due to the presence of connective tissue around the graft during the period of differentiation. Whether this influence should be considered as inductive in the usual sense of an organizer is questionable, since neither nervous tissue, notochord, nor mesodermal derivatives were formed. The connective tissue may merely provide a necessary set of stimuli, perhaps mechanical in nature, which activate the graft to the expression of certain ectodermal potencies already present in the cells.

Comparison of the results of differentiation of presumptive epidermis in the dorsal lymph spaces and in the optic cavity reveals the absence of ectodermal derivatives other than epidermis in the optic cavity. Since connective tissue also eventually surrounded grafts in the optic cavity, an apparent discrepancy exists between these two series. This is explained by the fact that the transplants were initially free in the optic cavity and came to be surrounded by connective tissue only after two or three days. Holtfreter (1938c) showed that presumptive ectoderm, isolated from inductive influences, soon loses its ability to react, even though it is subsequently placed in contact with an inductor. Hence the probable explanation of the results obtained here from culture of presumptive epidermis in the optic cavity is that the implanted rudiment had lost its competence for reaction before connective tissue grew in to surround the graft.

The series of transplantations of presumptive epidermis into muscles of the tail suggests, however, that inductive powers exist in this region of the larva.

The close correlation between degree of association of muscle and graft and the presence of nervous tissue or cartilage indicate that processes of induction by muscle may account for their appearance. Although the number of specimens in which notochord and muscle formed was too small to permit a similar relation to be established for these structures, muscle may also have been an inductive agency here. The possibility cannot be excluded, however, that the appearance of notochord and muscle was due to accidental inclusion of mesodermal anlagen in the grafts. This is rendered less probable by the failure of these derivatives to appear in ectodermal grafts in the optic cavity, dorsal lymph spaces, or brain in the present experiments; and by the experiments of Dürken, Kusche, and Bautzmann, in all of which notochord and muscle formed frequently from grafts of presumptive ectoderm in the optic cavity.

The nature of the inductive influences in the muscles of the tail, if present, has not been ascertained in these experiments. Specific chemical factors within the muscles, with or without physical agencies, may be the cause of these effects. Differentiation into nervous tissue, cartilage, and other structures may also conceivably be due to a nonspecific combination of physical conditions within the muscles of the tail, activating latent potencies of presumtive epidermis toward the structures noted. Such an effect would come under the heading of induction in the broader sense. Barth (1941) has shown *in vitro* that large explants of the presumptive epidermis of *Amblystoma punctatum* will form nervous tissue in the absence of an organizer, particularly if the anteroposterior axis of the explant is preserved during healing. No evidence is available from his results or those of other workers, however, to indicate that purely physical influences can cause differentiations of any structures other than ectodermal derivatives.

The great variation in structures produced, as well as in their shape and relative size, may be attributed to the complexity of the initial relation between graft and host. It was impossible to produce exactly the same type and extent of contact between muscle and graft in any two operations. The cut surfaces of muscle were always somewhat irregular, and the presence of a certain amount of fibrin and blood elements in the incision further complicated the relation. The incision closed tightly in some specimens, whereas in others there was a slight gaping of the cut. Moreover, the animal's movements after operation displaced the graft a certain amount. These observations appear sufficient to explain the fluctuations.

The question arises whether induction by the muscles of the tail, if it occurs, is to be regarded as a normal faculty residing in this region or whether the death of muscle cells through the operative procedure liberates substances which effect the inductions. The first alternative seems the more probable, since dead inductors in general produce nothing but medullary structures (Spemann 1938, p. 232) without organization into definite organs. In the present experiments, however, a variety of tissues was produced in addition to medullary structures, and even in the nervous tissue typical formations were occasionally obtained, such as spinal cord, ganglia, and parts of the brain with choroid plexus.

The regeneration blastema from the tail and limb of amphibians is said to be inducing, at least in the adult *Triton* (Umanski 1932, 1933); hence the possibility of induction by regenerating tissues in the tail must also be considered. Since the aggregation of regenerating cells was not noticeable here until four or five days after operation, the influences from this source may be disregarded. Holtfreter's results (1938c) reported above show that the reactive power of the ectoderm would be lost well in advance of the time when the inductive powers in regenerating cells could become effective.

The sequence of events following transplantation of presumptive epidermis into the cavity of the brain may best be understood by reference to Holtfreter's description (1938b) of the behavior of presumptive epidermis in salt solution. Typical differentiation of epidermis never occurred in his experiments unless connective tissue was also present in the explant. An atypical epithelium invariably formed from pure presumptive epidermis. In urodeles, the explant *in vitro* began to disintegrate after ten days; whereas in anurans disintegration probably began earlier.

Holtfreter's description parallels the phenomena observed in grafts in the brain cavity. Wherever connective tissue did not come into contact with the graft, differentiation into typical epidermis did not occur. This result, reached independently in the present experiments, is in accord with similar conclusions reached by Emerson (1941) on the development of presumptive epidermis in blastema mesenchyme. Separation of the cells of the graft within the cavity of the brain was seen at a time when disintegration, according to Holtfreter's observations, would have occurred in similar grafts *in vitro*. The fact that individual cells did not disintegrate immediately after separation suggests that favorable nutritive conditions were present in the ventricles of the brain. Prolonged existence of these atypical cells apparently was not possible, since they gradually disappeared in animals of increasing postoperative age.

The disappearance of grafts of presumptive epidermis in the brain cannot be attributed to the nature of the location, since grafts of presumptive notochord exhibited flourishing growth in the same place, nor to the character of the graft, since presumptive epidermis in other locations was not resorbed. Hence these data may help explain the ultimate fate of cells which are not exposed to inductive influences but are provided with satisfactory nutritive substances.

These experiments do not give any information concerning the inductive powers of the brain in advanced larval stages. The lack of positive results may be due to deficient contact between nervous tissue and graft. The coincidence between graft and nervous tissue was usually restricted to a narrow region on either side of the transplant, where contact had been established with cut edges of the thin roof of the brain.

Comparison of the growth of structures derived from presumptive epidermis in various locations reveals further points of interest. Vigorous growth was noted only in the tail, where inductive processes had occurred in the graft; elsewhere only a minor increase in the mass of the transplant was found.

Differentiation in optic cavity and dorsal lymph spaces was very slow; yolk platelets were present in the cells for a much longer time here than in grafts of presumptive epidermis in the tail, and a sharp increase in growth rate did not occur after resorption of the yolk. An interrelation between induction and the basic conditions necessary for growth of ectodermal structures is suggested.

The results of transplantation of presumptive epidermis reported here may be explained without postulating unknown factors in the fluids of the host or a special type of differentiation in the graft. The divergent results of other workers may be considered here briefly in their relation to the present experiments. Dürken (1925, 1926) in *Rana fusca*, Kusche (1929, 1930) in *Triton*, *Amblystoma*, and *Rana fusca*, and Bautzmann (1929*b*) in *Amblystoma*, report differentiation of presumptive ectoderm into medullary tissue, cartilage, and usually notochord and muscle in the optic cavity. Kusche did not attempt an explanation, but Dürken and Bautzmann believed that these structures were produced by self-differentiation of the graft, and denied the possibility of influence by the host. It might be suggested that the discrepancy between these results and those here presented is due to the different animal types employed. However, Bytinski-Salz (1929), Schotté (1930), and Holtfreter (1929*b*, 1931*b*, 1938*b*) agree that no major differences exist between amphibian embryos of different species with respect to the state of determination or the self-differentiating power of the various regions of the gastrula.

A more plausible explanation for the conflicting results may be found in differences in technique. All the previous investigators used a pipette for implanting the gastrular rudiments, so that the graft was placed deep in the optic cavity, where complicated physical contacts of the graft with cut ends of muscle, nerves, and blood vessels may take place. Since the muscles of the tail may be inductive, the possibility of either physical or chemical induction by the muscles at the base of the optic cavity cannot be ruled out in their experiments. At any rate, in the present experiments, when contact of the transplant with structures at the base of the optic cavity was avoided by the use of different technique, the grafts invariably formed only skin. A reasonable doubt is therefore cast on previous interpretations of these earlier results, and in particular on the views of Bautzmann, upon which his theory of "bedeutungsfremde" development is based.

As mentioned previously, Emerson (1941) postulates favorability of the new location plus release of the ectoderm from its normal environment in explaining the structures formed by gastrula ectoderm in the regeneration blastema of the tail. It is difficult to see how mere favorability of an environment could in itself, without induction or activation, cause the appearance of a variety of ectodermal derivatives, unless the potencies for the independent expression of those structures existed previously in the ectoderm. This assumption would be open to the same criticism as the "bedeutungsfremde" development of Bautzmann. Further, if the release of ectoderm from its normal environment can cause the formation of nervous tissue, cartilage, and other structures, it is noteworthy that such formations do not result from similar

grafts in salt solution or in the peripheral region of the optic cavity. Emerson does not consider the possibility of induction from the blastema environment on the ectodermal grafts, perhaps because he considers blastema mesenchyme to be an indifferent tissue which can itself be induced. On the other hand no clear evidence has yet been presented that the regeneration blastema is in fact indifferent to the point of reacting positively and definitely to embryonic inductors. The data presented by Emerson (1940) to support his conclusion that blastema cells may be induced to form ear vesicles are not thoroughly convincing, based as they are on slight differences in staining and the absence of yolk platelets in the vesicles. In the present experiments the disappearance of yolk platelets occurred irregularly, so that one part of a graft might be fully differentiated and free of yolk while in other parts of the graft the cells were still yolk-laden and embryonic in appearance. Until some stronger evidence is forthcoming with respect to the actual developmental indifference of the regeneration blastema, the possibility must be considered that the blastema mesenchyme, as well as the regenerating ends of muscle and other axial structures, may act as an inductor on implants of ectoderm. Induction is used here in a broad sense to include physical factors of support and activation as well as possible chemical mediators. The complicated possibilities of contact between host and graft tissues would provide ample variation in the action of the host tissues on the graft to explain the variety of tissues formed.

The variable results obtained by Holtfreter (1929a, 1929b, 1931a) must also be considered here. In the experiments reported in 1929, involving differentiation of gastrular anlagen in the dorsal lymph spaces and coelom of older larvae, Holtfreter found that presumptive epidermis and presumptive medullary plate were self-differentiating, respectively, in these two places. Later, Holtfreter (1931a) showed that in the coelom presumptive epidermis formed nervous tissue in about half of the experimental animals. In explaining his later results from transplantation of presumptive epidermis into the coelom, Holtfreter assumes the presence of unknown factors, either chemical or physical, in the coelomic medium, which assist in the realization of potencies toward nervous formation.

If Holtfreter's view is accepted, differentiation in his experiments would represent a unique type of induction, inasmuch as there is no other description to date of nervous induction by factors in solution alone, without physical contact of ectoderm with a substrate containing the inductor. Furthermore, if the coelomic fluid contains inductive factors, it would differ radically from the fluids in the dorsal lymph spaces and the optic cavity, both of which, it is concluded from the present experiments, are neutral in a morphogenetic sense. The possibility of contact with visceral organs may not be excluded in transplantation into the coelom. Holtfreter, in fact, described fusions of the graft with mesenteries, and even vascularization of the graft by the host blood vessels. Although the presence or absence of inductive power in the internal organs has not been established, the possibility of induction by these agencies, perhaps even by purely physical means, should be considered.

In transplants of presumptive epidermis into the coelom, Holtfreter (1929a)

briefly described differentiation of the graft into a vesicle of typical skin, epidermis forming an outer layer and a typical corium containing connective tissue and melanophores lying within. The corial elements were believed to be derived from presumptive epidermis. If this were true, a discrepancy would exist between the behavior of presumptive epidermis in the coelom and in the cavity of the brain. But the present experiments indicate that within the brain, connective tissue is not formed by the graft, and the graft eventually disintegrates in the absence of formative influences. Connective tissue seen in transplants in the coelom may conceivably be derived from the host. The isolated position of the graft observed at the time of fixation may not always have been maintained throughout the culture period; a temporary adhesion to structures in the peritoneal cavity, similar to the fusion Holtfreter describes, would suffice to permit the addition of connective tissue to the graft. Normal movements of the visceral organs could then easily dislodge the graft.

Connective tissue from the host may even appear in the vicinity of isolated objects in the coelom. This is indicated by Holtfreter, who showed that an isolated *Daphnia* shell in the peritoneal cavity was surrounded by connective tissue, and by Little (1929), who found that pieces of mammalian thyroid or gonad in the coelom of tadpoles were likewise encapsulated and infiltrated by connective tissue. Although these are pathological phenomena, not to be compared with the normal differentiation of skin found by Holtfreter, they serve to emphasize the caution which is necessary in ascribing the presence of connective tissue in coelomic implants of presumptive epidermis to differentiation in the cells of the graft.

SUMMARY

1. Differentiation and growth of selected anlagen from the early gastrula within the body of the older larva, together with the compatibility of these rudiments with the tissues of the host, have been studied by homoplastic implantation of presumptive notochord and presumptive epidermis into tadpoles of *Hyla regilla*. Grafts were placed in the empty optic cavity, in the dorsal lymph spaces, in the cavity of the brain, and within the muscles of the tail, to obtain variation in the degree and type of association between graft and tissues of the larva.

2. Presumptive notochord in the named locations differentiated into notochord, cartilage, muscles, nervous tissue, and a variety of other structures, all of which may be accounted for by the known regulatory powers of this region. These results correspond qualitatively with those reported by Holtfreter (1938b) for culture of this region in physiological salt solution. It is concluded that the larval environment does not affect the regulatory processes of differentiation occurring in isolated fragments of presumptive notochord.

3. No evidence was obtained that the embryonic organizer, as exemplified by presumptive notochord, is able to influence tissues of postembryonic age.

4. Differentiation of presumptive epidermis followed a variable course, dependent on the site of transplantation within the host:

In the optic cavity, skin formed as the sole product of differentiation of the

graft. Dermal structures appeared to be derived from connective tissue of the host, which eventually grew in to surround the implant.

Transplants in the dorsal lymph spaces formed skin, horny "teeth," sensory placodes, and atypical epithelial cysts. Formation of these structures may be correlated with the presence of connective tissue around the graft during the early phases of differentiation.

Implants into the cavity of the brain usually established a restricted connection with nervous tissue in the roof of the diencephalon or mesencephalon, whereas the major part of the graft projected freely into the ventricles below. Typical differentiation did not in general occur in this location. An atypical epidermis was formed, the cells of which eventually separated and disintegrated. Evidence is presented to indicate that the absence of connective tissue around the graft was accountable for the failure to differentiate into normal skin.

Grafts of presumptive epidermis placed within the muscles of the tail frequently differentiated into nervous tissue, cartilage, notochord, and olfactory epithelium. These structures occurred in almost all grafts in contact with or in close proximity to muscle fibers. Wherever parts of the graft had developed predominantly in the lymph spaces above the muscles, additional formations were found, similar to those observed in the dorsal lymph spaces.

5. On the basis of these experiments certain generalizations on inductive potentialities in the body of the larva are indicated:

a) The body fluids in the optic cavity and dorsal lymph spaces do not contain inductive factors, since grafts of presumptive epidermis in these locations form only epidermal derivatives when isolated from surrounding tissues.

b) Connective tissue may activate grafts of presumptive epidermis to form ectodermal structures in addition to epidermis. This influence, to be effective, must be exerted during the early phases of differentiation of the transplant.

c) The muscles of the tail may induce not only neural structures, but cartilage as well. Alternative explanations for development of ectodermal grafts in the tail are discussed.

6. These conclusions will suffice to explain all the facts of differentiation noted here, and obviate the necessity for postulating a special type of development in the graft or the presence of obscure factors in the body fluids. The divergent results and views of other workers have been critically analyzed in their relation to the present investigation.

7. The sequence of growth processes in grafts of presumptive notochord has been analyzed. It is suggested that this may best be explained by assuming that the graft is independent of its surroundings during the period of differentiation; later there is a strong subsequent influence from the host through which the tissues of the graft are brought into compatibility in physiological age with those of the larva.

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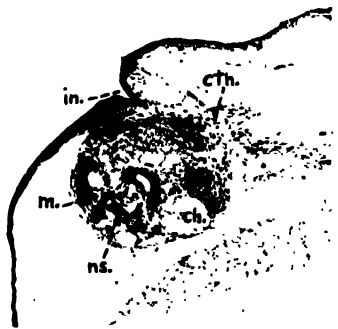
PLATES

PLATE 2

Fig. 2. OC 30–42. Transverse section through right optic cavity (×54). *ch.*, notochord; *cth.*, connective tissue of host; *in.*, indentation in conjunctival epithelium; *m.*, muscle; *ns.*, nervous tissue.

Fig. 3. OC 30 42. Detailed structures of the graft shown in figure 2 (×280). *ch.*, notochord; *cth.*, connective tissue of host; *m.*, muscle; *ns.*, nervous tissue.
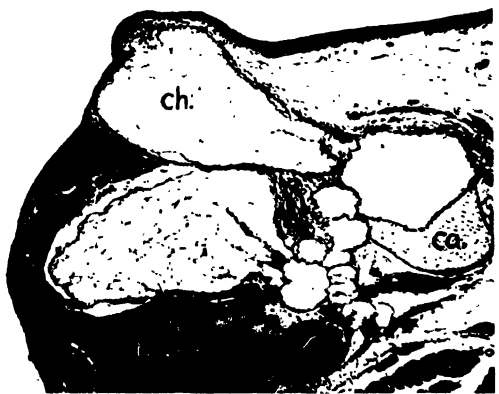
Fig. 4. OC 46 212. Transverse section through right optic cavity (×75). *ca.*, cartilage; *ch.*, notochord; *ns.*, nervous tissue.

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PLATE 3

Fig. 5. SC 44-21. Detailed view of ingrowth of notochord into nervous tissue (×565). *ch.*, notochord; *ns.*, nervous tissue.

Fig. 6. SC 90-211. Transverse section through dorsal lymph spaces (×99). *ca.*, cartilage; *ch.*, notochord; *cr.*, atypical epithelial cyst; *mh.*, muscles of host; *ns.*, nervous tissue; *sk.*, vesicle of skin.
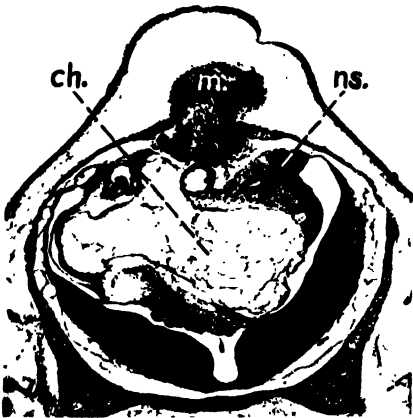
Fig. 7. BC 87-352. Transverse section through region of diencephalon (×32). *ch.*, notochord; *m.*, muscle; *ns.*, nervous tissue.
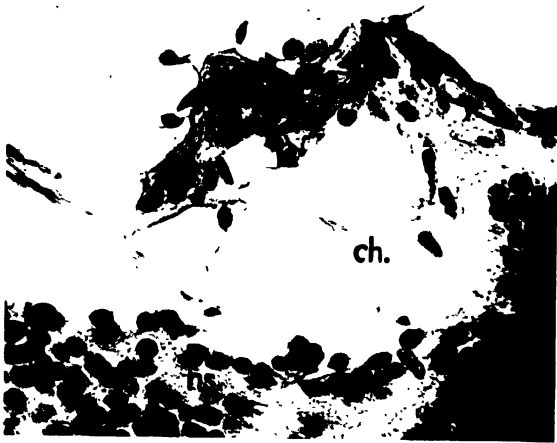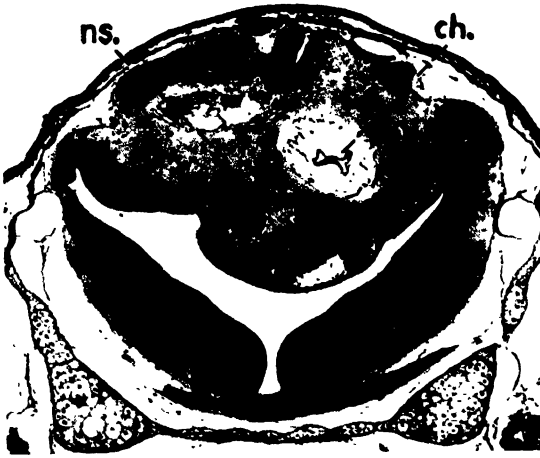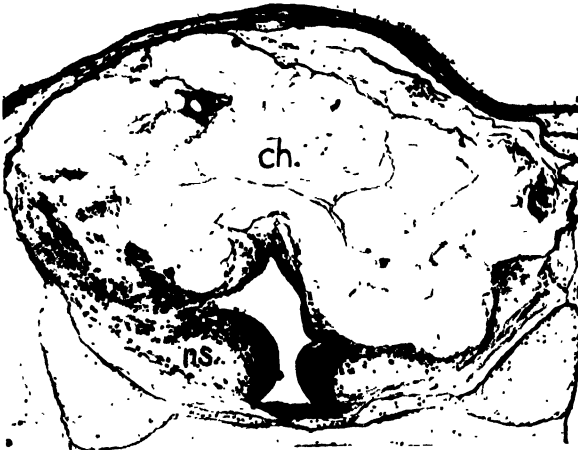
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PLATE 4

Fig. 8. BC 85 352. Transverse section through region of dien cephalon (× 54). *ch.*, notochord; *ns.*, nervous tissue.

Fig. 9. BC 44 632. Transverse section through region of dien-cephalon (× 66). *ch.*, notochord; *ns.*, nervous tissue.

Fig. 11. OE 51–191. Transverse section through right optic cavity (× 82). *cj.*, conjunctival epithelium; *ct.*, connective tissue of host; *sk.*, vesicle of skin.

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PLATE 5

Fig. 12. OE 51–191. Detailed structure of the graft shown in figure 11 (×280). *bm.*, basement membrane; *cj.*, conjunctival epithelium; *cp.*, epidermis; *pg.*, pigment.

Fig. 13. SE 53 362. Transverse section through dorsal lymph spaces (×280). *bm.*, basal membrane; *cp.*, epidermis; *sk.*, cyst of skin; *sp.*, sensory placode.

Fig. 14. SE 58–352. Transverse section through dorsal lymph spaces (×71). *bc.*, buccal epithelium; *l.*, lumen in buccal epithelium.
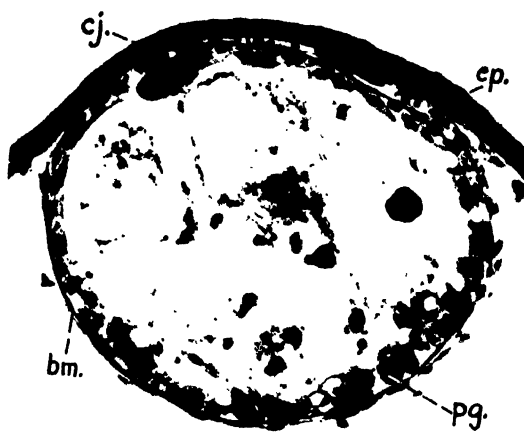
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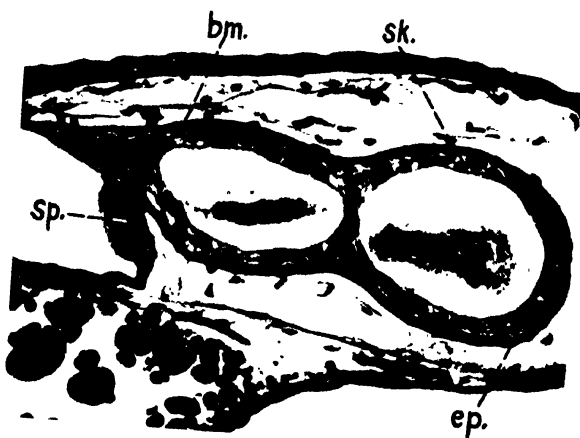
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PLATE 6

Fig. 15. SE 58 352. Detailed structure of graft shown in figure 14 (×280). *bc.*, buccal epithelium; *h.*, horny "teeth"; *l.*, lumen in buccal epithelium.

Fig. 16. BE 52 71. Transverse section through brain (×58). *gr.*, graft; *l.*, loose cells of graft in cavity of brain.

Fig. 17. BE 52–71. Detailed structure of graft shown in figure 16. *ct.*, connective tissue of host; *ep.*, epidermis; *s.*, fragmentation of cells of graft.

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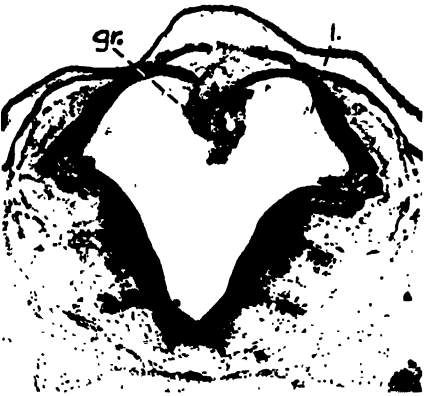
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PLATE 7

Fig. 18. ME 57–212. Transverse section through tail (× 63). *l.*, lumen in nervous tissue; *m.*, muscles of host; *ns.*, nervous tissue of graft.

Fig. 19. ME 54 212. Transverse section through tail (× 54). *gm.*, gray matter; *m.*, muscle fibers of host; *wm.*, white matter.

Fig. 20. ME 49 213. Transverse section through tail (× 285). *ca.*, cartilage; *l.*, lymph space; *m.*, muscles of host.

Fig. 21. ME 56–214. Transverse section through tail (× 63). *bc.*, buccal epithelium; *ch.*, notochord; *m.*, muscles of host.

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THE ORIGIN AND DIFFERENTIATION OF THE LARVAL HEAD MUSCULATURE OF TRITURUS TOROSUS (RATHKE)

BY

ARTHUR G. REMPEL

CONTENTS

THE ORIGIN AND DIFFERENTIATION OF THE LARVAL HEAD MUSCULATURE OF TRITURUS TOROSUS (RATHKE)

BY

ARTHUR G. REMPEL

INTRODUCTION

THE EARLY development of the cranial muscles of Amphibia has been the basis of a number of studies, of which those of Goette (1875) on *Bombinator,* Platt (1897) on *Necturus,* Edgeworth on the toad (1899) and on *Rana* and *Cryptobranchus (Menopoma)* (1935), and Piatt on *Amblystoma* (1938) are most inclusive. The adult musculature of *Triturus torosus* has been described by Smith (1927), and that of the closely related genus *Salamandra* was exhaustively treated by Francis (1934).
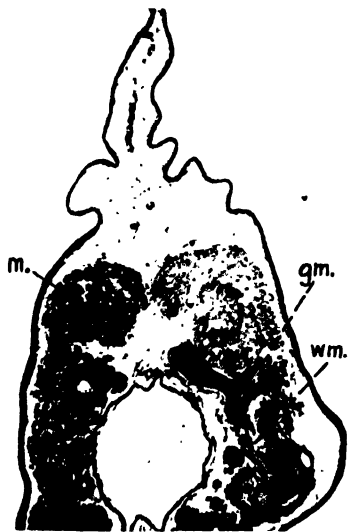
The purpose of the present paper is to supply a descriptive account of the development of the larval head muscles in a western newt, *Triturus torosus,* which has become an important laboratory animal on the Pacific Coast. The plan is (1) to trace each of the larval head muscles to its source in some rudiment of the early embryo; (2) to determine the time and the place within the embryo at which it separates from the remaining mesoderm as an independent muscle rudiment; (3) to follow the development of each such rudiment into a functional muscle; and (4) to describe briefly the condition of each muscle in the fully developed larva.

I wish to express my appreciation for the helpful criticism and suggestions of Professor J. Frank Daniel,* upon whose advice this study was undertaken.

MATERIAL AND METHODS

The embryos and larvae used in this study were obtained from ponds in the vicinity of Berkeley, California. Serial sections stained with Delafield's haematoxylin and eosin were prepared from numerous specimens ranging from neurulae to hatching larvae. Embryos and larvae used in dissection were fixed in corrosive sublimate acetic. Dissection proved to be the most satisfactory means of tracing muscle development, since it permitted the manipulation of individual muscles to determine their points of attachment and, by giving a three-dimensional picture, eliminated the necessity for reconstruction.

The nomenclature used is that adopted by Edgeworth (1935), who gave a comprehensive table of synonymy. The series of developmental stages 1 to 38, in terms of which the differentiation of the cranial muscles is described, has been kindly supplied to me by V. C. Twitty of Stanford University, who has prepared for *Triturus torosus* a series which closely resembles the correspond-

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* Since deceased.

ing series prepared by R. G. Harrison for *Amblystoma punctatum.* The key characters of stages 39 to 45 are, briefly :

Stage 39. Eye darkly pigmented anteriorly, dorsally, and posteriorly; light gray ventrally.

Stage 40. Eye black throughout with green color appearing dorsally; apex of anterior limb bud rounded.

Stage 41. Apex of anterior limb bud truncate, with indication of first two digits.

Stage 42. First two digits of hand well defined.

Stage 43. First two digits of hand diverging, hand slightly twisted.

Stage 44. Third digit of hand beginning to form.

Stage 45. Third digit of hand well developed.

ORIGIN OF THE MESODERM IN THE EMBRYO

The musculature of the body has its origin in mesoderm. In *Triton cristatus,* Hertwig (1881) defined two types of mesoderm, gastral mesoderm arising, as he thought, by a modified type of evagination from the walls of the archenteron, and peristomial mesoderm derived from the posterior portion of the embryo. Brachet (1902) retained the distinction between gastral and peristomial mesoderm, but believed that the gastral mesoderm in urodeles arises by delamination from the walls of the archenteron. The most complete work bearing on the origin of the mesoderm in Amphibia consists of the staining experiments of Vogt (1929) on *Triton* and *Pleurodeles* and shows that the mesoderm arises from the germ ring and is in a sense peristomial. In Vogt's map of presumptive organ rudiments in the early gastrula of urodeles the material destined to form the middle germ layer occupies a broad subequatorial band, bounded by presumptive ectoderm above and by entoderm below. The presumptive notochord forms a crescent lying in the dorsal portion of this band and extending downward toward the blastopore in the midline. Lateral, posterior, and ventral to the notochordal anlage lies the material of the somites. The lower margin of the mesoderm consists of presumptive lateral plate. In the dorsal midline the prechordal plate material lies between the chorda tissue and the entoderm. The tissue joining its sides with the first somites is the presumptive head mesoderm.

With the onset of gastrulation the first material to be invaginated is the entoderm of the foregut. This is immediately followed by the involution of the chordamesoderm along the dorsal lip of the blastopore. The first dorsal mesoderm thus to move over the lip of the blastopore lies close upon the entoderm and retains this connection until gastrulation is completed. The more posterior mesoderm, derived from the lateral and ventral lips, lacks contact with the entoderm and possesses a free anterior border. Stains applied to the various portions of the dorsal lip indicate that the material forming the notochord moves forward more slowly than does that forming the presumptive somites, and ceases to move anteriorly when the blastopore has become slit-shaped. The mesoderm continues its involution and is constantly replaced by tissue from the ventral and lateral portions of the blastoporal lip. It is thus pushed forward between the entoderm and the epidermis, with the older, more dorsal material in advance of the mesoderm arising from the ventral

part of the germ ring. Moreover, the mesoderm derived from the lateral and ventral lips converges toward the notochord, thus producing a thickening in the region of the future somites.

I have examined serial sections of gastrulae and neurulae of *Triturus* and find close agreement with the picture presented by Vogt.

CRANIAL MESODERM AND ORIGIN OF MUSCLE PLATES

Ever since Balfour's discovery of head cavities in the embryos of selachians much importance has been attached to these structures. Van Wijhe (1882) formulated the theory that the walls of these head cavities are homologous to the somites of the trunk and that the mesoderm within each subjacent visceral arch represents a corresponding portion of the lateral plate. Thus, in the shark there are three preotic somites, the premandibular, the mandibular, and the hyoid. In the branchial region the head somites themselves are transitory, whereas the portions of lateral plate associated with them remain as the mesoderm of the visceral arches. In elasmobranchs the mesoderm of the visceral arches is derived from a two-layered mesothelium which is divided into separate portions by the formation of the visceral clefts. The final result is the production of a mesodermal tube within each arch, the lumen of which lies in communication with the pericardial cavity.

This interpretation of the embryonic configuration of the head mesoderm of vertebrates has recently been challenged by Edgeworth (1935), who denies the existence of head somites dorsal to the visceral arches and considers only the premandibular somite of van Wijhe as valid. According to his theory, the mesodermal strips within the visceral arches are not lateral plate segments but are themselves homologous with the somites of the trunk, and their relation to the pericardium is directly comparable to the relation of the trunk somites to the peritoneal cavity.

Definite head cavities appear to be entirely lacking in the anurans (Goette, 1875; Corning, 1899). The literature on the urodeles is at variance. In *Triton*, Scott and Osborn (1879) described a series of well-defined epithelium-lined cavities within the visceral arches, together with a more anterior vesicle adjacent to the eye. Landacre (1921, 1926), working on *Amblystoma jeffersonianum*, described the head mesoderm as composed of forward extensions of the lateral plate which are divided by the visceral arches and supply the material for the visceral muscles. He states, "These lateral extensions contain cavities which in urodeles do not form definite head cavities or somites but their dorsal portions are undoubtedly homologous to the head somites of selachians." The somatic and splanchnic layers are held to retain their individuality until muscular differentiation begins.

In the embryo of *Necturus*, Platt (1897) described the visceral arches as solid mesodermal masses with only slight extensions of the pericardial cavity into the lower ends of the mandibular and hyoid arches. She states that dorsally the mandibular mesothelium is separated from that of the more posteriorly located arches and passes over directly into mesenchyme. A similar condition obtains in *Cryptobranchus*. Here the dorsal portion of the mandibu-

lar mesothelium is for a time continuous with the premandibular somite, and Edgeworth (1935) shows a premandibular somite composed of well-separated cells, suggestive of the mesenchymatous condition.

Piatt (1938) has not found cavities in the cranial mesoderm of *Amblystoma punctatum,* but recognizes three divisions of cranial mesoderm in the early embryo, as follows: (1) the prechordal mesoderm consisting of a "mandibular" segment and the mandibular muscle plate; (2) two additional head segments derived from the parachordal mesoderm adjacent to the notochord; and (3) the lateral plate which becomes separated into hyoid and branchial muscle plates.

ORIGIN OF THE MUSCLE PLATES IN TRITURUS TOROSUS

As indicated above, the early development of mesoderm in *Triturus* closely resembles the corresponding process in *Triton* and *Pleurodeles.* During gastrulation the mesodermal sheet moves forward over the entoderm with its dorsal portion well in advance of the ventral. This gives the mesoderm an oblique anterior margin. Anteriorly the prechordal plate lies close upon the archenteric roof. Laterally and posteriorly the mesoderm becomes separated from the entoderm as a layer of loosely arranged cells (stage 15, pl. 8, fig. 1), which passes over into the flanking material of the lateral plate. Owing to the curved surface of the early neurula, the more anterior dorsal mesoderm (*pm.,* pl. 8, fig. 1) grows down in front of the anterior margin of the lateral plate (*h.*). Its continued downward extension over the dorsal surface of the foregut, and the forward growth of the anterior border of the hypomere (*h.,* pl. 8, fig. 1), produce a well-defined cleft separating the prechordal plate and associated head mesoderm from the anterior margin of the lateral plate. This cleft, where entoderm and ectoderm remain in contact, is the site of the hyomandibular or first visceral pouch. Serial sections show that in the early neurula (stage 14) the prechordal mesoderm is a thin layer of cells still closely united with the anterior roof of the foregut; but as gastrulation continues this layer becomes more distinct. When the neural folds begin to rise (stage 17+) the prechordal mesoderm underlying the lateral portion of the medullary tissue in the anterior brain region forms two well-defined plates (*pm.,* pl. 8, fig. 2). A central mass of cells remaining between them then becomes the prechordal strand.

As the neural tube closes (stages 18 and 19, pl. 8, fig. 3) its lateral margins no longer cover the underlying prechordal mesoderm. This tissue now separates from the lining of the foregut and moves medially until it forms a compact mass lodged in the groove between the anterior portion of the closing neural tube and the roof of the foregut (pl. 8, figs. 3, 4). The band of prechordal mesoderm flanking the neural tube in the anterior brain region may henceforth be designated as the mandibular muscle plate (*mmp.,* pl. 8, fig. 5). It is significant that the tissue of the mandibular muscle plate is directly continuous with the parachordal mesoderm flanking the neural tube in the trunk region, and that its relation to the neural tube, as well as to the prechordal strand, is directly comparable to the relation of the mesoderm of

the somites to both the neural tube and the chorda. All this dorsal mesoderm may thus be regarded as epimeric in character as contrasted with the hypomere or lateral plate.

Shortly after the closure of the neural tube (stages 22 and 23, pl. 8, fig. 6; and pl. 9, fig. 7) the mandibular muscle plate is deflected to the rear, so that the extremity which was anterior comes to be directed posteriorly. The bend in the epimere occurs just behind the optic vesicle at the point where the primary cranial flexure of the brain is developing. There has thus resulted a reorientation of the prechordal mesoderm of the mandibular muscle plate, with its once anterior tip pointing posteriorly and forming the ventral end of the jaw-muscle rudiment.

As the mandibular muscle plate is shifted and delimited, cells are proliferated from its dorsal extremity (stages 23 and 24, pl. 9, figs. 7, 8). These cells continue to spread over the dorsal margin of the eye through the anterior region of the head (pl. 9, figs. 9–12). At the periphery of the mass they break up into mesenchyme, which eventually forms an enveloping mass of connective tissue about the brain. Posteromedial to the eye these cells remain somewhat more compact and in continuity with the mandibular muscle plate. They are the forerunners of eye muscles and have been termed the "premandibular somite" (*ps.*, pl. 10, figs. 14, 15).

The portion of the epimeric mesoderm from the upper end of the mandibular muscle plate to the first somite of the trunk also breaks up into mesenchyme. This process begins in the vicinity of the developing ear vesicle and extends posteriorly to the small first somite of the trunk. In front of the otic vesicle the dispersal of the epimere is delayed until stage 35 (pl. 9, fig. 12) is reached.

The first branchial pouch (*bp¹.*, pl. 9, fig. 8) perforates the lateral plate at or near stage 24 and delimits the anterior margin of the hypomere as the hyoid muscle plate (*hmp.*); the second pouch (*bp².*) is well formed by stage 27 (pl. 9, fig. 9), and marks off the first branchial muscle plate (*bmp.*); the third, separating the second branchial muscle plate from the hypomere remaining posteriorly, has reached the ectoderm by stage 30 (pl. 9, fig. 10); the last pouch penetrates the hypomere by stage 32 or 33 (pl. 9, fig. 11), dividing its posterior portion to form the third and fourth branchial muscle plates. There are thus formed a total of six mesodermal plates: the first, or mandibular, which arises from the anterior extremity of the epimere; and the hyoid and the four branchial muscle plates, which are formed in regular succession from the anterior portion of the lateral plate. Ventrally, the hyoid and branchial muscle plates are continuous with the walls of the pericardium. In *Triturus* there is never any indication that the lateral plate in the gill area becomes split into somatic and splanchnic layers. Shortly after the third and fourth gill pouches have pierced the lateral plate, the mesoderm within the branchial arches becomes somewhat spongy or mesenchymatous before clearly differentiated muscles make their appearance. The hyoid and mandibular muscle plates, however, remain as compact masses and may be traced directly into the muscles to which they give rise.

THE "PREMANDIBULAR SOMITE" AND THE ORIGIN OF
THE OCULAR MUSCLES

Corning (1899) was unable to determine the source or to follow the development of the ocular muscles in *Rana,* and Platt (1897) omitted these muscles in her account of the development of the cranial muscles in *Necturus.* Adelmann (1932) traced the rudiment of the ocular muscles of *Amblystoma punctatum* to a proliferation of cells from the prechordal plate. Although Edgeworth (1935) does not describe the source of the eye muscle rudiment, he regards the anlage of the ocular muscles in *Rana* and *Cryptobranchus* as a premandibular somite, which separates from the mandibular muscle plate at a relatively late period. Judging from his figures, this material in *Cryptobranchus* is mesenchymatous in organization. On the basis of work on *Rana* and *Cryptobranchus* as well as on other vertebrates, Edgeworth challenges the classical concept of the origin of the eye muscles, which is clearly stated by Neal (1918) and has been adopted for the urodele *Dicamptodon* by Eaton (1936). Edgeworth traces all the ocular muscles of the Amphibia to a single anlage, the "premandibular somite," the two obliques forming as anteriorly directed outgrowths of its dorsal and ventral ends, the *rectus externus* arising as a posteriorly directed outgrowth, and the other rectus muscles resulting by the division of the remaining portion of the somite. Piatt (1938) derives the two obliques and all the rectus muscles, except the *externus,* from what he terms the "mandibular" segment, which originates from the prechordal mesoderm. The *rectus externus* and *retractor oculi* are said to be outgrowths of the second and third segments of the head which come from parachordal material.

In *Triturus torosus* the rudiment of the ocular muscles is found in the mass of somewhat loosely arranged cells proliferated from the anterior margin of the upper end of the mandibular muscle plate. At first these cells are not distinguishable from those giving rise to the connective tissue enveloping the brain. With the enlargement of the eye and the brain, however, they become partly isolated from the more widely dispersed cells and fill the space between the eye, the infundibulum, and the anterior wall of the foregut. In this condition the mass may be recognized as early as stage 36, when it is still essentially mesenchymatous. Laterally it is continuous with the *levator mandibulae anterior* rudiment (*lma.,* pl. 9, fig. 12) of the mandibular muscle plate. The eye muscle rudiment or "premandibular somite" consolidates into a compact mass fitting close upon the posteromedial surface of the eye. It separates from the *levator mandibulae anterior* anlage at or near stage 38+, and, when the eye is removed, is seen to occupy the area behind the optic nerve with its thin anterior portion spreading forward (*ps.,* pl. 10, figs. 14, 15).

This forward spreading of the "somite" appears to represent the first step in the formation of the oblique muscles. By stage 39 the dorsal division of this migrating material (pl. 10, fig. 15) may be seen growing upward and forward in an arc over the internal surface of the eye. The forward growth continues until, in stage 41–, there is formed an arched band of cells extending upward

and anteriorly from the center of the "somite," then forward and down toward the anteroventral margin of the socket. The cells forming this outgrowth are larger than those of the underlying mesenchyme and have a slightly yellowish tinge. In some specimens it is impossible to recognize a compact outgrowth, and in these it appears that loosely arranged cells migrate in scattered formation to reach their destination at the locus of the future superior oblique. These migrating cells lie close upon the eyeball and tend to adhere to it. By stage 41+ the anterior tip of this band of cells has reached the anterior margin of the eye socket and becomes attached in the connective tissue between the telencephalic lobe and the olfactory cup. A break in the highest portion of the arc separates the anterior portion of the outgrowth as the superior oblique muscle. The basal, upwardly directed portion of the band is still continuous with the remainder of the "somite" and represents the rudiment of the superior rectus muscle. Below the optic nerve there is a more massive forward growth of the material. Pressing close under the optic stalk the tip of this forward growth soon reaches the anterior margin of the orbit behind the olfactory cup. This distal portion then separates as the inferior oblique.

The rest of the "somite" forms a thick crescentic mass lying upon the anterior surface of the *levator mandibulae anterior* and extending forward under the optic stalk. It separates to form the rectus muscles after histological differentiation is well advanced (stage 41−).

OBLIQUUS SUPERIOR (KLEIN, 1850)

Shortly after its separation from the dorsal outgrowth of the "premandibular somite" in stage 41− the rudiment of the superior oblique (*os.*, pl. 11, fig. 18; and pl. 12, figs. 21, 23) is transformed into muscular tissue. The lower, anterior tip of the outgrowth becomes anchored in the connective tissue behind the cerebral lobes, and the upper end becomes attached to the anterodorsal portion of the internal surface of the eye.

In the fully developed larva the superior oblique originates from the trabecula in the anterior portion of the orbit and curves outward in a posterodorsal direction to its insertion on the dorsal margin of the eyeball, just anterior to the insertion of the superior rectus. Its spreading fibers give the muscle the form of a broad, somewhat curved fan, the width at the point of insertion equaling almost half the length of the muscle.

OBLIQUUS INFERIOR (KLEIN, 1850)

When the inferior oblique muscle (*oi.*, pl. 11, fig. 18; and pl. 12, fig. 23) separates from the ventral outgrowth of the "premandibular somite" in stage 41−, it lies in the groove between the brain and the posterior margin of the nasal cup. Differentiation into muscle tissue proceeds at once. As in the *obliquus superior*, the distal tip of the outgrowth forms the origin of the muscle and becomes attached to the trabecular cartilage immediately below and slightly behind the superior oblique. The insertion is derived from the point at which the rudiment separates from the rest of the "somite" and soon

becomes attached to the ventral margin of the eye, just anterior to the *inferior rectus.*

In the older larva this muscle passes outward in a posteroventral direction. It is about a third shorter than the superior oblique and only about one-half as wide at the insertion.

RECTUS INFERIOR (KLEIN, 1850)

Upon the separation of the inferior oblique rudiment the horizontal portion of the "premandibular somite" separates from the rest (stage 41) to form the *rectus inferior* and *rectus internus (rin., ri.,* pl. 11, fig. 18; and pl. 12, fig. 23). Before this occurs, fibers develop within the mass and become attached to the trabecular cartilage, just behind the optic stalk. The separation begins distally (stage 42), and then extends toward the origin upon the trabecular cartilage. The posterior, the more ventral of the divisions, is the beginning of the *rectus inferior,* and the anterodorsal part forms the *rectus internus.* This common origin persists in a diminishing degree until it is lost in the later larval period. In the early larva the fibers of the two muscles cross each other at the point of junction, those of the inferior rectus passing over the base of the internal rectus. The insertion of the *rectus inferior* is at first relatively far back upon the posteroventral portion of the eyeball, and the direction of its fibers is predominately laterad and ventrad. As development continues, its insertion becomes shifted anteriorly so that in the older larva the muscle passes obliquely outward, forward, and downward to a point just behind the inferior oblique. The muscle has the shape of a broad fan, narrowing to a band near its origin.

RECTUS INTERNUS (KLEIN, 1850)

The *rectus internus (ri.,* pl. 11, fig. 18; and pl. 12, fig. 23) is also derived from the horizontal portion of the "premandibular somite," and represents the anterior dorsal division of this tissue. It lies in front of the *rectus inferior* and its distal portion reaches the inferior oblique rudiment, with which it was previously continuous. The attachment at the point of origin lies just posterior to that of the *rectus inferior.* When first formed, the fibers of the internal rectus pass outward in an anteroventral direction. Then a shifting of the insertion from the ventral margin to the internal surface of the eye gives the muscle a horizontal direction and results in crowding the base of the optic stalk upward. With further elongation of the muscle its insertion is shifted onto the anterior margin of the eye, between the two oblique muscles. Thus, in the 35-millimeter larva this muscle has the form of a narrow attenuated fan, curving over the internal surface of the eye. It is then the longest of the ocular muscles.

RECTUS SUPERIOR (KLEIN, 1850)

The superior rectus *(rs.,* pl. 11, fig. 18; and pl. 12, figs. 21, 23) is derived from the upper vertical portion of the eye-muscle rudiment at the point where this tissue gave rise to the superior oblique. Its upper disal portion, forming the insertion, was previously continuous with the superior oblique. The proximal portion of the rudiment tapers to a point and, serving as origin, gains an attachment upon the trabecular cartilage a little above and posterior to the

origins of the inferior and internal recti. When the fibers become differentiated within this rudiment, they lie in a predominantly vertical direction, tending a little forward as they approach their insertion. In the older larva the muscle retains its original orientation, passing upward and forward as it approaches its insertion on the posterodorsal margin of the eye, just behind the superior oblique. It is of medium size, a little smaller than the two preceding muscles, and fan-shaped, as are the other ocular muscles.

RECTUS EXTERNUS (KLEIN, 1850)

The external rectus muscle (*re.,* pl. 11, fig. 18; and pl. 12, fig. 23) separates from the posteroventral portion of the "premandibular somite" (stage 41+). At this time the muscle has the form of a cone, with its base, the future insertion, applied to the posterior portion of the internal surface of the eye. The apex of the cone, forming the origin, is directed inward and lies deep between the other rectus muscles and the *levator mandibulae anterior.* In stage 42, as fibers develop in this muscle its direction is almost straight outward. It becomes attached upon the trabecular cartilage a short distance posterior to the inferior and internal rectus muscles. Although it is at first conical, the external rectus soon acquires the flattened fan-shaped form of the other eye muscles. With the enlargement of the eye its insertion is carried backward and slightly downward, so that in the older larva the muscle bends over the surface of the *levator mandibulae anterior* as it passes outward to its final attachment, in the posterior margin of the eye.

Shortly after separating from the other rectus muscles (stage 43) the external rectus gives rise to the *retractor oculi.*

RETRACTOR OCULI (STANNIUS, 1846)

In *Triturus torosus* the *retractor oculi* (*ro.,* pl. 11, fig. 18; and pl. 12, fig. 23) is formed from the more dorsally situated fibers of the *rectus externus* which separate from the parent muscle at stage 43 and constitute about half of the entire rudiment. Though at first both divisions have similar proportions, the *retractor oculi* retains its original insertion upon the posteromedial surface of the eyeball and fails to take part in the elongation characteristic of the external rectus. This difference becomes particularly striking after stage 46.

In the later larval period the *retractor oculi* increases greatly in breadth, extending its insertion in a crescentic area around the optic nerve. It lies within the ring of the rectus muscles extending from the dorsal margin of the *rectus externus* around the optic nerve to the anterior margin of the *rectus inferior.* It originates just below the orbital fissure, and is the most massive of the ocular muscles.

THE MANDIBULAR MUSCLE PLATE AND ITS MUSCLE DERIVATIVES

The development of the mandibular muscle plate in the frog was briefly described by Corning (1899), and in the toad its differentiation into muscles was observed by Edgeworth (1899). Platt (1897) gave an account of its differenti-

ation into jaw muscles in *Necturus* but did not deal with its earlier origin. She states that it is ventrally continuous with the pericardium through the mediation of the hyoid muscle plate, and that the pericardial cavity extends into the lower ends of both plates. The embryological source of this tissue in urodeles has been traced to the prechordal mesoderm by Adelmann (1932). His conclusion has been corroborated by Starck (1937) and Piatt (1938), and it applies equally to *Triturus,* as is shown in this paper. The differentiation of the mandibular muscle plate into the muscles of the jaw has been described by Edgeworth (1925, 1935) in *Cryptobranchus* and by Piatt (1938) in *Amblystoma.*

In *Triturus torosus* we have seen the derivation of the mandibular muscle plate from the prechordal epimeric mesoderm of the neurula. By the time the embryo has entered the tail-bud stages the apex of the plate has grown posteriorly and ventrally over the surface of the foregut (*mmp.,* pl. 8, figs. 5, 6; pl. 9, figs. 7–10) until it has reached the anterior margin of the hypomere (stage 28, *mmp.,* pl. 9, fig. 9), and it continues to grow posteriorly so as to overlap the anterior portion of the pericardium. In *Triturus torosus* there is no indication that the material of the mandibular muscle plate ever comes into morphological continuity either with the walls of the pericardium or with the hyoid muscle plate, as is asserted of *Necturus* by Platt (1897). In the tail-bud stages it is covered by a layer of cells derived from the neural crests.

The differentiation of the separate muscle rudiments from the mandibular muscle plate begins during the tail-bud stages (pl. 9, figs. 10–12). As the muscle plate elongates and grows posteriorly and ventrally toward the heart (pl. 9, figs. 9, 10) its tip consists of the material for the two intermandibularis muscles. In the midline the ventral tip of the muscle plate merges with its counterpart from the opposite side. The fused intermandibularis rudiment now begins to widen and flatten, and it continues to do so until after it has differentiated into muscular tissue (*imp.,* pl. 9, fig. 12; pl. 10, figs. 13–16). At stages 38+ and 39 the ventral portion of the mandibular plate separates from the dorsal portion, which now becomes the masticatory muscle plate, and the procartilaginous rudiment of the mandible becomes insinuated between the two divisions. The formation of the intermandibularis rudiment is accompanied by a turning of the masticatory muscle plate, which brings its posterior margin outward and the anterior margin in under the optic cup. At the same time the muscle rudiments derived from the masticatory plate are set apart.

The first indication of the *levator mandibulae externus* (*lme.,* pl. 9, fig. 12; pl. 10, fig. 14; and pl. 12, fig. 22) may be seen in stage 32. A swelling on the posterior margin of the muscle plate develops just dorsal to the intermandibularis anlage and somewhat lower than the level of the eye. This swelling becomes enlarged into a prominent ridge and its upper tip becomes distinct in stage 35 (pl. 9, fig. 12). Above this muscle rudiment the posterior margin of the muscle plate is at first continuous with the epimere in the ear region, and, like the latter, breaks up into mesenchyme. The lower extremity of the *externus* is delimited when the intermandibularis rudiment is cut off in stage 39–.

The rudiment of the *levator mandibulae posterior* arises from the deeper portion of the same cell mass which superficially forms the *levator mandibulae externus* (*lme.*, pl. 9, fig. 12). On removal of the latter the *levator mandibulae posterior* is seen as an independent anlage as early as stage 39, when its upper and lower limits approximately coincide with those of the *externus*. But the *posterior* is smaller, more nearly vertical in position, and tapers to its upper end. Both these rudiments lie posterior and external to, and somewhat lower than, the *levator mandibulae anterior*.

A thickening arises upon the anterior margin of the mandibular muscle plate at or near stage 28, and by stage 30 (pl. 9, fig. 10) it forms a well-defined anteriorly directed process lying just behind the optic stalk. This outgrowth is the lower extremity of the *levator mandibulae anterior* and is most distinct in stages 37 and 38+ (*lma.*, pl. 10, figs. 13, 14), when its lower surface touches the sides of the oral evagination (*oe.*). The *levator mandibulae anterior* as a whole arises from the anterior margin of the masticatory muscle plate. Its anterior margin remains in continuity with the "premandibular somite" until stage 38.

INTERMANDIBULARIS POSTERIOR (DRÜNER, 1901)

Arising from the ventral apex of the mandibular muscle plate, the substance of the intermandibularis muscles originally (pl. 8, figs. 1, 2) lies under the transverse fold of the neural plate. As the muscle plate grows down over the surface of the foregut, this ventral tissue joins the corresponding rudiment from the other side. The intermandibularis anlage separates from the masticatory muscle plate when it has already become noticeably widened (stage 39–), so that its posterior margin slopes caudad at an angle of about 35 degrees. The anterior margin touches the edge of the jaw rudiment, which now occupies an almost transverse position. When fibers begin to develop within the intermandibularis tissue (stage 41–), most of them come to lie parallel to its posterior margin and meet with those of the opposite side at an angle in the ventral midline. They constitute the *intermandibularis posterior* (*imp.*, pl. 9, fig. 12; pl. 10, figs. 13–16; pl. 11, figs. 18, 19; and pl. 12, figs. 22–24). A small group of short, nearly transverse fibers along the anterior margin belongs to the *intermandibularis anterior* (*ima.*, pl. 11, fig. 19). The *intermandibularis posterior* becomes attached to the medial margin of the mandible, extending from the narrow *intermandibularis anterior* along all but the posterior third of the jaw.

A fascia upon which the fibers become inserted begins to develop in the midline (stages 42–43), remaining narrow posteriorly but gradually widening in its anterior portion. With its origin upon the mandible and its insertion on this median fascia, the muscle in its final form consists of a flat sheet of somewhat posteriorly directed fibers.

INTERMANDIBULARIS ANTERIOR (DRÜNER, 1901)

The *intermandibularis anterior* (*ima.*, pl. 11, figs. 19, 20; and pl. 12, figs. 24, 25) arises from the most anterior portion of the intermandibularis rudiment and becomes isolated as an individual muscle upon the differentiation of its

fibers (stage 41–). These fibers span the symphysis of the developing jaw and are not interrupted by a median fascia. The muscle forms a narrow but compact band which unites the anterior extremities of the mandibular rami in the larva and is retained until metamorphosis.

LEVATOR MANDIBULAE EXTERNUS (EDGEWORTH, 1925)

The rudiment of the *levator mandibulae externus* (*lme.*, pl. 9, fig. 12; pl. 10, figs. 13–15; pl. 11, figs. 17, 18; and pl. 12, figs. 21, 22) is derived from the outer posterior margin of the masticatory muscle plate. In frontal section an incipient separation between the constituents of the plate may be recognized as early as stage 38+, and by stage 39 the medial surface of the muscle is so clearly delimited that in dissections the rudiment may easily be separated from the underlying tissue. Originally (stage 39–) the *externus* has the form of a vertical oblong mass, two and a half times as long as its diameter and of approximately circular cross section. Its lower end, formerly continuous with the intermandibularis anlage, now touches the posterior end of the developing jaw. The upper tip is surrounded by mesenchyme and is separated from the otic vesicle by a distance equaling almost the entire length of the rudiment. Then the rudiment begins to tilt toward the rear, and to grow in length and width until (stage 41–) its upper end is in contact with the anterolateral surface of the otic vesicle. Ventrally the muscle becomes inserted upon the proximal portion of the mandible.

During the early larval stages the *levator mandibulae externus* is considerably flattened, and its lower end remains so until the later larval period. It inserts upon the external surface of the jaw somewhat anterior to the articulation with the quadrate. The upper portion becomes more massive and spreads backward and dorsally until its origin covers the entire anterolateral surface of the otic capsule.

LEVATOR MANDIBULAE POSTERIOR (EDGEWORTH, 1925)

Together with the preceding muscle, the *levator mandibulae posterior* (*lmp.*, pl. 12, fig. 23) is derived from the lower portion of the masticatory muscle plate. In frontal section its rudiment may be recognized in stage 39+, when it is a mass of tissue barely separated from the *levator mandibulae externus,* which flanks it laterally, and the *levator mandibulae anterior,* which lies anteromedial to it. Upon removal of the *levator mandibulae externus* the *levator mandibulae posterior* is seen as a small vertical mass, tapering dorsally where it presses against the posterolateral surface of the *levator mandibulae anterior.* On further development the muscle elongates, becomes somewhat flattened, and assumes a sloping position.

In the larva it lies against the anterior surface of the quadrate originating from the anterior surface of the otic capsule. It inserts upon the upper surface of the mandible, proximal to the *l. m. externus* and just in front of the articulation. This is the smallest of the larval muscles concerned with closing the mouth; in the older larva it hardly equals one-fourth the bulk of the *l. m. externus.*

LEVATOR MANDIBULAE ANTERIOR (EDGEWORTH, 1925)

The *levator mandibulae anterior (lma.,* pl. 9, fig. 12; pl. 10, figs. 13–15; pl. 11, fig. 18; and pl. 12, figs. 21–23) separates from the inner anterior margin of the masticatory muscle plate in stage 39–. It then occupies a vertical position behind the eye anteromedial to the *levator mandibulae posterior.* The dorsal end is somewhat pointed and reaches higher than the other levators of the jaw; it becomes clearly outlined when the neighboring tissue, forming the upper tip of the muscle plate, disperses to form the mesenchyme. After its separation from the muscle plate, the more massive lower section of the rudiment begins to grow down behind the oral evagination, toward the rudiment of the mandible. In the young larva the muscle increases slightly in bulk and relative length, and widens at its origin in the connective tissue surrounding the trabecular cartilage. In the older larva it becomes very massive, and though its shorter fibers spring from the posterior alisphenoid region, the muscle extends its origin medially and posteriorly over the surface of the cranium until it reaches the *intertransversarius capitis superior.* From there this largest of the jaw muscles extends forward and laterad from near the midline to the orbit, then abruptly downward over the anterior surface of the otic capsule, where, joining its shorter fibers, it descends to a fleshy insertion on the medial surface of the mandible, somewhat anterior to the articulation.

THE HYOID MUSCLE PLATE AND ITS MUSCLE DERIVATIVES

The early condition and subsequent differentiation of the hyoid muscle plate, like that of the mandibular, was studied in the Anura by Corning (1899) and Edgeworth (1899). For urodeles this tissue and its derivatives were first described by Platt (1897). The work of Adelmann (1932), Starck (1937), and Piatt (1938) shows that this muscle plate has a fundamentally different origin from that of the mandibular arch, and that in common with the branchial mesoderm it is derived from the anterior portion of the lateral plate. The differentiation of the hyoid mesoderm in *Rana* and *Cryptobranchus* has been described by Edgeworth (1935); and Piatt (1938) has given an account of its development in *Amblystoma.*

In *Triturus torosus* the hyoid muscle plate is derived from the anterior margin of the hypomere (*h.,* pl. 8, figs. 1–6; and pl. 9, fig. 7). During the period of neurulation this anterior margin loses its originally jagged contour (pl. 8, fig. 1), and with the formation of the first branchial pouch (*bp¹.,* pl. 9, fig. 8) is set off from the remainder of the lateral plate. Ventrally the hyoid muscle plate (*hmp.,* pl. 9, figs. 8, 9) is at first in continuity with the walls of the developing pericardium (*p.,* pl. 9, fig. 10), and dorsally it joins the epimere. The presomital epimere in the region of the auditory vesicle (*av.,* pl. 9, fig. 10) soon disperses to form mesenchyme, and at stage 32 a condensation of mesenchymal cells at the upper end of the hyoid muscle plate connects this structure to the ventrolateral surface of the auditory vesicle (pl. 9, figs. 11, 12).

The lower end of the muscle plate now grows down over the anterior surface of the pericardium until it meets its counterpart from the opposite side. At

the same time the muscle plate as a whole becomes flattened into a widening band (stages 35–38, pl. 9, fig. 12; and pl. 10, figs. 13, 14). The upper end, which anchors the hyoid mesoderm upon the otic vesicle, is not involved in the flattening process but remains circular in cross section. It separates from the flattened part of the muscle plate (stage 39, pl. 10, fig. 15) as the rudiment of the *depressor mandibulae* (*dm.*), and its lower end acquires a free, pointed, somewhat anteriorly directed tip (pl. 10, figs. 13–15). After separating from the depressor rudiment, the major portion of the hyoid muscle plate shifts its upper attachment onto the dorsal end of the rudiment of the first ceratobranchial cartilage. It continues to broaden (pl. 10, figs. 15, 16) and becomes divided to form the *branchiomandibularis* (*bm.*), *branchiohyoideus externus* (*bhe.*), *interhyoideus* (*ih.*), and *interhyoideus posterior* (*ihp.*).

Histological differentiation slightly precedes the division of the hyoid muscle plate into separate muscles, and when these become visible they may be recognized by the position in which their incipient fibers lie. The *interhyoideus* (*ih.*), the first muscle to form from the lower portion of the hyoid plate, separates from the anteroventral portion of the muscle plate at stage 39 (pl. 10, figs. 15, 16). Laterally its posterior fibers lie at an angle to those forming in the upper portion of the plate, and as the ceratohyoid cartilage is formed the *interhyoideus* becomes attached to its distal end. The remaining portion of the plate, with its attachment upon the first ceratobranchial rudiment, forms a sheet of fibers lying posterior and internal to the *interhyoideus*. Division into separate muscles begins at once by a process of splitting between the fibers which originate from the first ceratobranchial. This splitting commences at the ventral ends of the fibers but is delayed near their origin.

In *Triturus torosus* the material of the *depressor mandibulae* (*dm.*, pl. 10, figs. 13–15; pl. 11, figs. 17–20; and pl. 12, figs. 21, 22, 24, 25) is apparently formed by an aggregation of mesenchyme cells derived from the parachordal epimeric mesoderm lying under the developing ear. This muscle forms a distinct mass as early as stage 37 (pl. 10, fig. 13) and splits off obliquely from the wider part of the hyoid muscle plate in stage 38+ (pl. 10, fig. 14), whereupon its ventral tip immediately begins to grow down and forward until it reaches the mandibular anlage at stage 39 (pl. 10, fig. 15).

At first gently tapering from its origin upon the posterolateral surface of the otic vesicle, the rudiment soon acquires a uniform diameter throughout its length and inserts upon the lower and medial surface of the posterior end of the mandible. During larval life it increases somewhat in relative size and spreads back over the side of the otic vesicle as far as the first *levator. arcus* (*la.*, pl. 12, fig. 22). It runs almost parallel to the *levator mandibulae externus*.

Although in many genera of urodeles this muscle has a temporary function as a levator of the hyoid arch, in *Triturus torosus* it becomes attached to the mandible from the beginning.

INTERHYOIDEUS (DRÜNER, 1901)

Early in stage 40 the *interhyoideus* (*ih.*, pl. 10, figs. 15, 16; pl. 11, figs. 18, 19; and pl. 12, figs. 22–24) is a narrow ribbon of incipient fibers converging toward

the tip of the chondrifying ceratohyal and passing ventrally to the midline. As the differentiation of the fibers continues, the lower portion of the muscle begins to broaden and in the midline becomes extended far forward, internal to the *intermandibularis posterior*.

In the larva the muscle has the form of a broad triangular fan consisting of a flat layer of loosely associated fibers which originate a little below the tip of the ceratohyal and insert along the linea alba, partly internal and partly posterior to the *intermandibularis*. Since the tip of the ceratohyal is considerably below the surface of the skin the muscle passes outward between the *branchiomandibularis* and the *branchiohyoideus externus* to reach its external distribution within the gular fold.

INTERHYOIDEUS POSTERIOR (EDGEWORTH, 1931)

The *interhyoideus posterior* (*ihp.,* pl. 10, figs. 15, 16; pl. 11, figs. 18, 19; and pl. 12, figs. 22–24) is perhaps more appropriately called the *gularis* (Eaton, 1937). At the time of its origin from the lower section of the hyoid muscle plate the *interhyoideus posterior* consists of a narrow band of rudimentary fibers lying just behind the *interhyoideus*. Laterally these fibers converge and become attached somewhat below the upper end of the first ceratobranchial cartilage. With further development the fibers of the *interhyoideus posterior* become separated from each other below, thus greatly increasing the width of the muscle.

In the larva the muscle retains its origin on the ventral margin of the first ceratobranchial just below the attachment of the *branchiohyoideus externus*. A few of its fibers arise lower than, and anterior to, the rest along the length of the cartilage. Ventrally the radiating fibers of this fan-shaped muscle insert upon the linea alba, just behind the *interhyoideus* and upon the margin of the gular fold.

BRANCHIOHYOIDEUS EXTERNUS (EDGEWORTH, 1935)

Above the *interhyoideus posterior* the incipient fibers arising within the hyoid muscle plate also attach to the ceratobranchial cartilage. They cut diagonally across the upper end of the muscle plate and end freely along its anterior margin, where they become inserted upon the tip and along the posterior margin of the lateral portion of the ceratohyal, which at this stage lies in a transverse position immediately anterior to the muscle plate. The upper fibers, which become attached to the tip of the ceratohyal, consititute the rudiment of the *branchiomandibularis* (*bm.*). The rest, inserting upon the posterior surface of the cartilage, make up the *branchiohyoideus externus* (*bhe.,* pl. 10, figs. 15, 16; pl. 11, figs. 18–20; and pl. 12, figs. 22–25). The *branchiohyoideus externus* now shifts its insertion medially along the posterior border of the ceratohyal cartilage, the median portion of which grows forward with the development of the jaw. To span the distance between the now widely separated attachments, the *branchiohyoideus externus* increases greatly in length until, in the older larva, it becomes a large ribbonlike muscle attached to the posterior border of the ceratohyal and passes posterolaterad and then pos-

terodorsad between the *branchiomandibularis* and *interhyoideus posterior,* to its attachment upon the tip of the first ceratobranchial (pl. 12, figs. 22–25). Its lower, anterior portion is covered by the *interhyoideus.*

<div align="center">BRANCHIOMANDIBULARIS (EDGEWORTH, 1935)</div>

Eaton (1936) regards the *branchiomandibularis* (*bm.,* pl. 10, fig. 15; pl. 11, fig. 18; and pl. 12, figs. 21–23) as only a subdivision of the *depressor mandibulae,* whereas in *Triturus torosus* it has an independent embryological origin. It arises from the upper margin of the hyoid muscle plate after the separation of the *depressor* (stage 39+), and its fibers lie parallel to and just above those of the *branchiohyoideus externus.* Dorsally the fibers become attached to the upper end of the first ceratobranchial cartilage; ventrally they insert on the distal extremity of the ceratohyal. As the *branchiohyoideus externus* shifts to a more median attachment upon the hyoid bar its lower end becomes somewhat separated from the *branchiomandibularis,* but posteriorly the two muscles remain almost as a continuous sheet. The ventral attachment of the *branchiomandibularis* is secondarily shifted from the hyoid bar to the posterior angle of the mandible (stage 43).

In the older larva this ribbonlike muscle remains attached to the tip of the first branchial arch, above the *branchiohyoideus externus,* although a few of its fibers become shifted onto the lateral surface of the *depressor mandibulae.* It then passes forward and ventrad to the vicinity of the ceratohyal, where its fibers converge into a tendon which inserts medially to the *depressor mandibulae* upon the base of the mandible.

<div align="center">THE BRANCHIAL MUSCLE PLATES</div>

According to Noble (1931), the muscles associated with the branchial arches of Amphibia arise from mesenchyme, whereas most of the literature traces their embryological origin to compact mesothelia or muscle plates situated between the branchial pouches. Platt (1897) described the development of the branchial muscles of *Necturus,* deriving the *depressor mandibulae* (*digastricus*) and the *levatores arcuum* from a "dorsal mesothelium" above the visceral pouches, and the intrinsic gill muscles and *subarcuales* (*constrictores arcuum*) from the mesothelium lying within the arches themselves. Edgeworth (1899, 1911, 1920, 1935) traced all the muscles associated with the branchial skeleton of Amphibia to the muscle plates lying within the embryonic visceral arches. Landacre (1921, 1926) also ascribes a mesothelial origin to the branchial muscles of *Amblystoma jeffersonianum,* and Piatt (1938) determined the derivatives of the branchial muscle plates in *Amblystoma* by experimental procedure.

In *Triturus torosus* the lateral plate mesothelia, which come to lie within the four branchial arches, differ from the hyoid muscle plate in that they lose their compact arrangement and apparently give rise to a loose aggregation of mesenchymal cells before differentiating into muscular tissue (pl. 9, fig. 12; and pl. 10, fig. 13). There is also a dispersal of the parachordal mesoderm flanking the side of the neural tube dorsal to the visceral arches. In the ventral

region mesenchyme is proliferated from the walls of the pericardium, adjacent to the branchial area, where the ventral muscles of the gill arches make their appearance. Thus, the muscles of the branchial arches and gills, though ultimately derived from the mesothelium of the gill region, do not form directly from solid plates of mesoderm but from aggregations of cells within the mesenchyme derived from this material. This agrees with the statement given by Noble (1931).

<div align="center">LEVATORES ARCUUM I–IV (FISCHER, 1864)</div>

The *levatores arcuum (la.,* pl. 10, figs. 14, 15; pl. 11, figs. 17, 18; and pl. 12, figs. 21–23) arise in the mesenchyme dorsal to the visceral pouches and posterior to the rudiment of the *depressor mandibulae.* The first *levator* appears as an independent strand of undifferentiated cells in stage 38+. It lies external to the glossopharyngeal ganglion a short distance behind the *depressor mandibulae* and is directed downward and a little anteriorly into the upper end of the first branchial arch. *Levatores II–IV* have a common beginning in a comparatively massive condensation of mesodermal cells dorsal to the posterior gill pouches and external to the vagus ganglion. Almost as soon as it is distinctly formed (stage 38+, pl. 10, fig. 14) the lower margin of this mass is parted into three ventrally directed processes, the first of which is most distinct and represents the lower end of *levator arcus II.* The ventral portions of the other two *levatores arcuum* are only very indistinctly indicated, but they become more clearly separated in stage 39 (pl. 10, fig. 15) when the muscle fibers differentiate. At the same time a few fibers separate themselves from the posterior median surface of *levator arcus IV* to form the rudiment of the *cucullaris.*

All the *levatores arcuum* increase in length and become attached to the posterior surface of the otic vesicle, with the origin of *levator arcus I* somewhat lateral to that of the others. The lower ends of the muscles insert upon the upper tips of the branchial cartilages as these become differentiated. Elongation of the first arch carries the insertion of *levator arcus I* posteriorly so that its final direction is down, laterad, and backward. Its final insertion is shifted to a subterminal position on the lateral face of the first ceratobranchial, where it is covered by the *branchiomandibularis,* and its massive origin is carried a little dorsad upon the surface of the otic capsule. The other *levatores,* particularly the last, which spreads far down upon the fourth arch, also shift their insertions to a lower position upon the ceratobranchials. They are arranged in a palmately radiating series originating in common just anteromedial to *levator arcus I* and immediately lateral to the posterior portion of the first segment of the *intertransversarius capitis superior.*

<div align="center">CUCULLARIS (RYLKOFF, 1924)</div>

The *cucullaris* or *trapezius (c.,* pl. 12, fig. 23) is the only branchiomeric muscle associated with the pectoral girdle. It becomes separated from the posteromedial surface of the last *levator arcus* in stage 39, when the differentiation of fibers is in progress. Whereas the anterior ends of the *levatores arcuum II, III,*

and *IV* grow forward to become attached to the otic vesicle, the upper end of the *cucullaris* rudiment does not appreciably alter its position and its fibers spread to the side of the dorsal trunk musculature. The posterior portion of the rudiment remains fairly compact and grows downward and somewhat posteriorly in the direction of the developing limb bud and reaches the pectoral girdle at or near stage 45. In the older larva (pl. 12, fig. 23) the muscle consists of a strong band of fibers spreading slightly at its origin upon the posterior surface of the skull and the fascia of the second myotome of the *intertransversarius capitis superior*. The muscle narrows slightly as it reaches its insertion upon the pectoral cartilage at the junction of two elements of the girdle, the scapula and the procoracoid.

LEVATORES BRANCHIARUM I–III (FISCHER, 1864)

Edgeworth derives the *levatores branchiarum* (*lb.*, pl. 11, figs. 17, 18; and pl. 12, figs. 21–23) from the "lower portions" of the branchial muscle plates. A more complete statement is given by Platt (1897, p. 442) who in *Necturus* derives both the *levatores* and *depressores* of the gills from mesothelial muscle bands within the gill arches.

In *Triturus torosus* it has not been possible to follow the mesoderm of the visceral arches unmistakably into the substance of the intrinsic gill muscles, and it appears that these muscles arise from mesenchymal cells which migrate into the gill buds and differentiate into muscular tissue a little later.

The *levatores branchiarum* are fairly distinct by stage 39, at which time they have already reached their final orientation. They are then represented by only a few fibers, but these soon become more numerous and the muscles elongate as growth and development continue. In the larva the *levatores branchiarum* are well-developed attentuated bands, fraying out somewhat as each muscle inserts upon the skin of the median or posterior surface of one of the gills. Their origins are upon the branchial arches, the first arising slightly below the tip from the posterior surface of the second ceratobranchial, whereas the other two arise in common from a cartilaginous bridge connecting the tips of the third and fourth arches.

DEPRESSORES BRANCHIARUM (FISCHER, 1864)

Like the preceding set of muscles, the *depressores branchiarum* (*db.*, pl. 11, figs. 19, 20; and pl. 12, figs. 22–25) in *Triturus torosus* appear to be derived from mesenchyme cells which migrate into the gill buds from the mesodermal bands of the visceral arches. They may be recognized in stage 39 as fine threads of fibers extending posteriorly from the central portions of the first three ceratobranchials into the lower halves of the three external gills. Throughout all but the last part of the larval period they increase in strength and assume the form of narrow bands whose lower fibers insert within the proximal portion of the external gills, whereas the upper fibers extend farthei back, some of them reaching the tip of the gill. The origins of these three muscles are upon the median third of the first three ceratobranchials along their lower or posterior margins.

THE SUBARCUALES

The subarcuales muscles are derived from the mesoderm of the lower ends of the four branchial arches lateral to the pericardium. This tissue has been described as a mesothelium in *Necturus* (Platt, 1897) and as muscle plates in *Cryptobranchus* (Edgeworth, 1920), denoting in each a certain compactness and unity within the mass, even before its transformation into muscle.

In *Triturus torosus* the tissue corresponding to the branchial muscle plates appears to be more loosely arranged and to fit more properly the designation of mesenchyme. In dissection this condition is shown by the translucent character of the tissue and the lack of cohesion between the individual cells. This stands in contrast to the dense tissue making up the hyoid and mandibular plates. The *subarcuales* appear to arise as condensations within this material and may first be recognized as muscle rudiments in stage 38+ (*so., sr. IV,* pl. 10, fig. 14).

SUBARCUALIS RECTUS I (EDGEWORTH, 1920)

This is the first of the *subarcuales* (*sr.,* pl. 10, fig. 16; pl. 11, figs. 19, 20; and pl. 12, figs. 24, 25) and the only one retained as a functional muscle after metamorphosis. It may be recognized in stage 38+, where it arises as a compressed mass of cells lying within the basal portion of the first branchial arch. By stage 39 (pl. 10, fig. 16) it has acquired the form of a stout, clearly defined spindle, whereas its axis, which originally lay almost in a transverse position, has been directed a little more anteriorly. At this time also there occurs a marked forward growth of the medial end, giving the rudiment an abruptly bent form and greatly increasing its total length. The growth of the muscle brings its anterior tip in touch with the posterior margin of the hyoid rudiment, a little lateral to the midline, and when the hyoid cartilage moves forward the muscle becomes straightened and comes to lie primarily in the longitudinal direction.

The anterior portion of the *subarcualis rectus I,* inserting upon the base of the ceratohyal, becomes tendinous. The rest of the muscle remains a slightly flattened, spindle-shaped structure arising from the proximal end of the first ceratobranchial, just anterior and medial to the attachment of the *subarcualis rectus IV.*

SUBARCUALES OBLIQUI II AND III (EDGEWORTH, 1920)

The early rudiments of these larval muscles (*so.,* pl. 10, figs. 14, 16; pl. 11, figs. 19, 20; and pl. 12, figs. 24, 25) are formed within the lower ends of the second and third branchial arches and extend medially to the sides of the pericardium. When first visible (stage 38+, pl. 10, fig. 14) they lie embedded within dense mesenchyme, apparently arising as condensations of mesenchyme cells. Their medial ends are then directed forward, and are closely approximated. Upon further development, fibers arise within the rudiments which increase in length and grow anteriorly. Originally ending at the side of the pericardium, the median ends of the *subarcuales obliqui* become shifted onto the sides of the first segment of the *rectus cervicis* as this muscle broadens and spreads dorsally over the surface of the pericardium. Their larval origin re-

mains upon the fascia covering the first segment of the *rectus cervicis*. From their origin on the fascia of the *rectus cervicis* the two *obliqui* extend posteriorly and slightly upward and laterad. The anterior portions of the *obliqui* become combined into a single muscle which is greatly flattened as it passes over the surface of the *rectus cervicis* and becomes rounded in cross section as it extends posteriorly and laterally. The lateral portions are separate, *subarcualis obliquus II* inserting upon the base of the second ceratobranchial, and *subarcualis obliquus III* attaching upon the corresponding point of the third arch.

SUBARCUALIS RECTUS IV (EDGEWORTH, 1920)

In stage 38+ (pl. 10, fig. 14) the *subarcualis rectus IV* (*sr. IV*, pl. 10, figs. 14, 16; pl. 11, figs. 19, 20; and pl. 12, figs. 24, 25) is a loosely constructed strand of cells lying superficial to the *subarcuales obliqui* rudiments and medial to the lower ends of the visceral pouches, and extending forward from the base of the fourth arch to the rudiment of the first ceratobranchial. In general terms the muscle rudiment has then already reached its permanent orientation, which suggests that its forward growth, described by Edgeworth (1920, 1935), has (in *Triturus torosus*) taken place before the cells uniting to form it have definitely become associated. There is very little change other than a further consolidation of the rudiment until stage 40–, when, at the level of the second gill pouch, a small strip of cells splits away from the lateral surface of the muscle. This represents the slip to the second gill arch. Early in stage 41 it has been completely separated, and from the lateral surface of the posterior portion of the rudiment another division, that going to the third arch, has been split off. All divisions of the muscle increase in size. In older larvae they originate in common from the proximal portion of the fourth ceratobranchial. The most medial slip inserting upon the base of the first ceratobranchial, just behind the attachment of the *subarcualis rectus I,* is considerably thicker than the shorter slips; the second passes to the proximal portion of the second ceratobranchial; and the smallest and outermost attaches to a corresponding but slightly more distal point on the third arch. All three parts are rounded in cross section and attach to the ventral margins of the branchial cartilages.

TRANSVERSUS VENTRALIS IV (EDGEWORTH, 1920)

Edgeworth (1920, 1935) states that the *transversus ventralis IV* (*tv.*, pl. 10, fig. 16; pl. 11, fig. 20; and pl. 12, fig. 25) is derived, in common with the *subarcualis rectus IV,* from the lower part of the mesoderm lying within the fourth branchial arch, and arises as a medially directed outgrowth of this material between the pericardium and the floor of the pharynx. A similar origin is assigned to this muscle by Piatt (1938) in *Amblystoma.* In *Triturus torosus* in stage 38– serial sections show that the dorsal surface of the pericardium is still closely applied to the ventral wall of the pharynx. By stage 38+ a thin layer of cells has come between these structures. These cells represent the rudiment of the *transversus ventralis IV* and, like the cells forming the *subarcuales,* have already reached approximately their final orientation. Definite elongation of the cells becomes evident at stage 39. The rudiment then

consists of a relatively narrow band reaching from the ventral portion of the fourth branchial arch, where it is closely associated with the posterior end of the *subarcualis rectus IV*, in a medial and slightly anterior direction to the midline between the pericardium and the pharynx. Its median portion lies in front of the rudiment of the larynx. As muscular differentiation continues, the median portion of the *transversus ventralis* increases in width until its more anterior fibers come to be directed forward at an angle of nearly 45 degrees. The posterior fibers retain their original transverse orientation. The outer ends of most of the fibers which make up the *transversus ventralis* become attached along the ventral margin of the basal portion of the fourth ceratobranchial, near the *subarcualis rectus IV*, but the more posterior fibers become anchored in the connective tissue behind the fourth ceratobranchial. The larval muscle consists of a heavy, roughly triangular sheet of spreading fibers. Their median ends insert upon the linea alba and their divided lateral attachment results in a slight separation of the two divisions at their origin.

DILATOR LARYNGIS (GÖPPERT, 1894)

Edgeworth (1920) derives the *dilator laryngis* (*dl.*, pl. 10, fig. 16; pl. 11, fig. 20; and pl. 12, fig. 25) and the *laryngei* from mesenchyme proliferated by the dorsal wall of the pericardium. Thus he ascribes to them an embryological origin fundamentally different from that of the other muscles associated with the visceral arches, which he regards as of myotomal origin, coming from cephalic myotomes or muscle plates. Piatt (1938) distinguishes the muscles of the larynx by ascribing to them a splanchnic origin in contrast to the derivation of the gill-arch muscles from the somatic mesoderm of the lateral plate.

In *Triturus torosus* a number of somewhat elongated cells, which lie within the mesenchyme between the pericardium and the anterior end of the esophagus, and which extend upward around the lateral margin of the esophagus, represent the beginnings of the *dilator laryngis* (stage 38+). This muscle arises in close association with the posterior fibers of the *transversus ventralis IV*. It may be distinguished as a separate structure in stage 39, consisting of a narrow band of rudimentary fibers which extends from the mesenchyme surrounding the larynx to the side of the esophagus, where it swings upward and ends in the connective tissue between the skin and the myotome. The lateral portion of the muscle grows slightly more dorsad, whereas the lower median portion grows inward over both the dorsal and ventral surfaces of the larynx to meet in the midline. The medial continuation of the muscle represents the two *laryngei*, but at first they are not visibly separated from the *dilator laryngis*. Later a raphe appears between them and the *dilator;* but this is difficult to distinguish.

In the older larva the dorsal end of the *dilator laryngis* originating from the second segment of the *intertransversarius capitis superior* is flattened and somewhat broader than the rest of the muscle. The remainder of the muscle is ribbonlike. It passes downward over the dorsal portion of the *cucullaris* and then turns inward under the esophagus, lying close against the posterior margin of the *transversus ventralis*.

LARYNGEUS VENTRALIS (EDGEWORTH, 1920) AND LARYNGEUS DORSALIS
(EDGEWORTH, 1920)

These small muscles (*lv.* and *ld.,* pl. 12, fig. 25) are at first continuous with the parted medial portion of the *dilator laryngis.* They may be recognized as discrete entities in stage 43 or 44, and together take the form of a depressed ring surrounding the larynx. A raphe separating these muscles from the *dilator laryngis* appears to develop gradually and is not clearly demonstrable until the larval period. Through larval life the *laryngeus ventralis,* which forms the lower portion of the ring, spans the gap left between the medial ends of the *dilatores* and covers the ventral surface of the larynx.

The *laryngeus dorsalis* is somewhat narrower than the *laryngeus ventralis,* with which it originates at the base of the *dilator*—whence it continues medially and dorsally to insert upon the upper or posterior surface of the larynx. Near their origin both muscles appear to be closely bound together.

The *constrictor laryngis* was not observed.

THE OCCIPITAL SOMITES AND THEIR MUSCLE DERIVATIVES

A few of the head muscles in urodeles are derived from typical postotic somites. They represent the anterior extremities of the longitudinal segmental muscles of the trunk, which find their anterior attachment upon the cranium and the visceral skeleton. The development of the occipital portion of the trunk muscles in urodeles has been fully described by Goodrich (1911), who gives an excellent series of reconstructions. Maurer (1891) described the development of the *rectus abdominis,* the anterior portion of which is here included as the *rectus cervicis.* Platt (1897) described both the early development of the occipital muscles and the formation of the hypobranchial spinal muscles of *Necturus.* In *Amblystoma,* Lewis (1910) showed the relation of the myotomes to the ventrolateral muscles, and Piatt (1938) gave a full account of the development of the hypoglossal musculature. Detwiler (1922) and, especially, Adelmann and Maclean (1934*a,* 1934*b,* 1935) have studied the entire anterior spinal region experimentally and have supplemented our knowledge of the normal formation of the hypobranchial muscles with data bearing on the equipotentiality and regulative ability of the somites from which they are derived.

The position of the material for the first four somites in the blastula of the salamander has been determined by Vogt (1929). In *Triturus torosus* the demarcation of actual somites may be observed at the time of the formation of the neural plate (stage 16, pl. 8, fig. 1) as two or three narrow furrows in the parachordal mesoderm. These furrows deepen and are soon followed by others which are more caudally situated. The first three well-defined somites thus formed are the source of five conspicuous muscles of the head. Anterior to them a smaller mass of parachordal mesoderm becomes delimited at or near stages 24 to 27, when the material lying still farther forward gives rise to mesenchyme. It represents the first postotic somite (s^1., pl. 9, fig. 8) but forms only a few muscle fibers, most of which degenerate in the larval period.

From the time of its formation the first myotome lies closely applied to the anterior surface of the second, whereas beginning at or near stage 28 the latter tends to grow forward over the first. By stage 30 (pl. 9, fig. 10), as the cells of the myotomes are elongating into fibers, a small dorsal portion of the first myotome becomes separated from the rest and closely associated with the anterodorsal tip of the second. A faint line of demarcation between them appears to remain until stage 33, but is then lost, making the first segment of the dorsal musculature double in origin. A horizontal division of the first myotome arises at the level of the vagus nerve rudiment. By stage 38+ this split is carried into the second segment, and in early larval life extends through the third as well. Above it lies the anterior portion of the *intertransversarius capitis superior,* which during middle larval life gives rise to a fan-shaped deeper lying *rectus capitis posterior* from its lower portion. Below the vagus and the first spinal nerves lies the *intertransversarius capitis inferior* (*ici.,* pl. 9, fig. 12).

The rudiments of the hypobranchial musculature appear in stage 28. They are ventrally directed outgrowths (*vp.,* pl. 9, fig. 10) of the lower ends of the second, third, and fourth myotomes. The first of these soon grows in advance of the other two, and the last tends to lag and is slightly broader than the rest. All three grow downward behind the gill area and anterior to the pronephros (pl. 9, figs. 10–12; pl. 10, fig. 13), thus being separated from the more caudal processes arising from the other myotomes. When these ventral processes have passed the gill area their lower portions become broadened. This broadening is just visible in stage 37, but becomes more marked in the succeeding stages (pl. 10, figs. 13–15) until the *rectus cervicis,* to which the ventral processes give rise, is fully formed. At the same time the ventral processes become isolated from the myotomes by the degeneration of their narrow connections and grow forward under the branchial area and over the lateral surface of the pericardium, finally to attach upon the urobranchial cartilage. In stage 39– an outgrowth from the lateral surface of the anterior segment commences to grow forward toward the rudiment of the mandible to form the *geniohyoideus* (*gh.,* pl. 10, fig. 16).

Because Edgeworth does not discuss the occipital muscles of urodeles, I shall follow Francis (1934) in the terminology pertaining to the anterior differentiations of the longitudinal trunk musculature, though several striking differences exist between the extent and the composition of two of these muscles as they occur in the adult *Salamandra* and the larval *Triturus torosus.*

THE OCCIPITAL MUSCLES

INTERTRANSVERSARIUS CAPITIS SUPERIOR (GAUPP, 1896)

The *intertransversarius capitis superior* (*ics.,* pl. 9, fig. 12; pl. 10, figs. 13–15; pl. 11, figs. 17, 18; and pl. 12, figs. 21–23) is derived from the dorsal division of the first four myotomes and represents the anterior portion of the dorsal trunk musculature. Its first segment is compound, having been formed by fusion of the upper parts of the first two myotomes in stage 34. Its fibers begin

to differentiate in the tail-bud period. At first (stage 24) the myotomal rudiments of this muscle lie well behind the auditory vesicle above the posterior gill area. But long before they are split away from the *intertransversarius capitis inferior* rudiment their fibers, particularly in the first segment, become elongated and the muscle extends forward on each side of the hind brain until by stage 38 its somewhat pointed anterior tip has passed over the posterodorsal surface of the ear. Meanwhile, the separation from the *intertransversarius capitis inferior* rudiment has extended through the myotome of the third somite, isolating two segments of this muscle. After stage 42 the anterior portion of the *intertransversarius capitis superior* increases in width and relative mass and its lower median fibers give rise to the *rectus capitis posterior*. In its final larval condition the *intertransversarius capitis superior* is a massive bulging band of muscular tissue arising from the myoseptum between the third and fourth vertebrae and inserting over the entire posterodorsal surface of the otic capsule. It consists of three segments and is divided by two myosepta.

RECTUS CAPITIS POSTERIOR (NISHI, 1916)

The *rectus capitis posterior* (*rcp.*, pl. 12, fig. 21) arises as a special differentiation of fibers derived from the *intertransversarius capitis superior* and split from the undersurface of its medial margin. In middle larval life, just before the hind limb bud shows indications of digits, the *rectus capitis posterior* makes its appearance as a thin fan of fully formed fibers originating in common from the tip of the first neural arch and spreading widely to insert upon a diagonally descending line along the posterior surface of the chondrocranium and just under the lower fibers of the *intertransversarius capitis superior*. In older larvae the *rectus capitis posterior* gradually increases in strength; its fibers become more numerous, finally constituting a fairly dense triangular sheet.

INTERTRANSVERSARIUS CAPITIS INFERIOR (GAUPP, 1896)

The *intertransversarius capitis inferior* (*ici.*, pl. 9, fig. 12) arises from the ventral division of the occipital myotomes the separation of which from the *intertransversarius capitis superior* is finally completed at or near stage 45. Like the dorsal division, it becomes elongated and, growing forward lateral to the chorda, its narrow, pointed tip reaches the level of the otic vesicle by stage 38. At the time of its formation and during early larval life this muscle consists of four segments. In the later larval period there follows a progressive degeneration of its attentuated anterior tip, effecting at first the loss of only the apical myotome but finally involving the next one as well. The third of the original myotomes forms the permanent anterior insertion of the muscle. This inserts upon the posterior surface of the chondrocranium, ventral and somewhat lateral to the foramen magnum. The myoseptum separating the third and fourth myotomes degenerates. The *intertransversarius capitis injerior* originates upon the myoseptum associated with the second vertebra, beyond which point the muscle continues into the trunk, dorsally as the lower portion of the *dorsalis trunci*, and ventrally as the *subvertebralis*.

THE HYPOBRANCHIAL MUSCLES

RECTUS CERVICIS (EDGEWORTH, 1928)

The *rectus cervicis* (*rc.*, pl. 10, figs. 14–16; pl. 11, figs. 19, 20; and pl. 12, figs. 24, 25) is derived from the ventral processes (*vp.*, pl. 9, figs. 9–12; and pl. 10, fig. 13) of the second, third, and fourth myotomes. Muscular differentiation begins at or near stage 38+, when the muscle consists of a thin and rather narrow band of incipient fibers lying upon the lateral surface of the pericardium and extending forward to the level of the second branchial pouch. Owing to the forward spreading of the ventral processes of the more caudally situated myotomes, the *rectus cervicis* is now continuous posteriorly with the rudiments of the ventrolateral abdominal musculature. At this stage also it gives rise to a dense, conical, anteriorly directed outgrowth from the anterior margin of the first segment. This is the rudiment of the *geniohyoideus* (*gh.*, pl. 10, fig. 16). It then continues to grow forward, reaches the base of the first branchial arch by stage 40, and, upon the formation of the urobranchial rudiment, becomes inserted in the angle between the urobranchial cartilage and the first branchial arch. There then ensues a gradual increase in the mass of the muscle. It spreads upward along the side of the pericardium and as a thin sheet extends ventrally over the lower surface, but it fails to reach the midventral line. The anterior extremity grows forward with the cartilages upon which it inserts. In the older larvae the *rectus cervicis* is a massive structure, comma-shaped in cross section, becoming thinner as it extends over the ventral surface of the pericardium. It inserts principally within the angle between the first hypobranchial and basibranchial cartilages, and to a lesser extent along the sides and posterior tip of the urobranchial. The muscle is traversed by two myosepta. Although it is commonly considered as the anterior portion of the *rectus abdominis,* in the larva of *Triturus* only its mesial fibers correspond in direction to those of the *abdominis,* whereas in its lateral portion the upward spreading fibers of the *rectus cervicis* continue uninterruptedly into the oblique fibers of the lateral body wall.

GENIOHYOIDEUS (CARUS, 1828)

The rudiment of the *geniohyoideus* (*gh.*, pl. 10, fig. 16; pl. 11, figs. 19, 20; and pl. 12, figs. 24, 25) arises in stage 38+ as a proliferation from the tip of the anterior segment of the *rectus cervicis* rudiment. It is then a small conical mass of cells pointing forward in the direction of growth. This rudiment rapidly increases in length but fails to broaden greatly at its base. As its narrow anterior tip grows forward, its base becomes separated from the tip of the *rectus cervicis* and is shifted onto the external surface of that muscle, being left behind, as it were, by the advancing parent muscle. The forward-growing tip of the *geniohyoideus* reaches the mandibular rudiment, adjacent to the symphysis at stage 40. It soon acquires the thickness of the more basal portion and after stage 41 the entire muscle begins to flatten into a narrow ribbonlike structure. With the development of the urobranchial rudiment the

base of the *geniohyoides* becomes shifted medially onto the posterior tip of this cartilage. Throughout the larval period the *geniohyoideus* is a narrow, flattened, band-shaped structure lying just lateral to the midline and uniting the tip of the mandible with the tip of the urobranchial cartilage.

SUMMARY

1. In the Pacific Coast Newt, *Triturus torosus,* the larval head muscles are traced to their early rudiments in the embryo, and the differentiation of each rudiment into a functional muscle of the larva is described.

2. The material giving rise to the cranial muscles is derived from three sources: the prechordal mesoderm, the lateral plate, and the postotic somites. The relation of these areas to Vogt's map of presumptive areas for the urodele is pointed out.

3. The prechordal mesoderm extends forward from the somites. In the neurula it may be recognized as a distinct layer of cells overlying the anterior portion of the archenteric roof, beneath the lateral portions of the neural plate. It forms the mandibular muscle plate which, among other things, gives rise to the following muscles of the jaw: *levator mandibulae anterior, levator mandibulae posterior, levator mandibulae externus, intermandibularis anterior,* and *intermandibularis posterior.*

4. The primordium of the eye muscles is derived from a group of cells proliferated from the anterodorsal portion of the mandibular muscle plate and for a time remains in continuity with it. It gives rise to the complete set of ocular muscles, including the *retractor oculi,* which is a derivative of the external rectus muscle.

5. The anterodorsal portion of the lateral plate, overlying the pharyngeal area, becomes perforated by the branchial pouches and is the source of the muscle plates of the hyoid and branchial arches.

a) The hyoid muscle plate differentiates directly into the following muscles: *depressor mandibulae, branchiomandibularis, branchiohyoideus externus, interhyoideus,* and *interhyoideus posterior.*

b) The branchial muscle plates break up into mesenchyme before muscular differentiation begins.

The *levatores arcuum* arise as condensations of this mesenchyme dorsal to the pharyngeal pouches.

The *cucullaris* is derived from the fourth *levator arcus.*

The *levatores branchiarum* and *depressores branchiarum* develop *in situ* within the gills. They are derived apparently from cells of the branchial muscle plates.

The *subarcuales* are derived from mesoderm lying within the lower ends of the four branchial arches, and in close proximity to the lateral portion of the pericardium. The *subarcualis rectus I* is formed within the first arch, and *subarcuales obliqui II* and *III* arise at the base of the second and third arches.

The *subarcualis rectus IV* when first observable lies in the mesenchyme lateral to the pericardium, extending forward from the fourth arch. It subsequently divides into three parts.

The *transversus ventralis* is formed from a thin mesodermal layer which becomes insinuated between the pericardium and the floor of the pharynx.

c) Mesenchyme derived from the lateral plate in the region of the pericardium is the source of the muscles of the larnyx.

6. The somites of the occipital region give rise to a third group of muscles associated with the head. These are special differentiations of the anterior trunk musculature, and in the larva they comprise three muscles in the occipital region and two in the hypobranchial area.

a) The hypobranchial group is formed from ventrally directed outgrowths of the second, third, and fourth postotic somites. In the larva it includes the *rectus cervicis* and the *geniohyoideus*, which are outgrowths from the first segment of the *rectus cervicis* rudiment.

b) The occipital muscles arise directly from the myotomes of the occipital region. They include the *intertransversarius capitis superior,* the *rectus capitis posterior,* and the *intertransversarius capitis inferior.*

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PLATES

PLATE 8

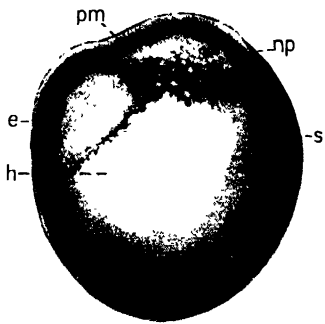Early formation of somites and mandibular muscle plate of *Triturus torosus* (stages 15–22).

Fig. 1. Early neurula after removal of epidermis and neural plate from its left side, showing prechordal mesoderm, lateral plate, and first indication of somites in stage 15.

Figs. 2–4. Prechordal mesoderm, lateral plate, and somites in neurulae of stages 17+, 19, and 20; lateral aspect.

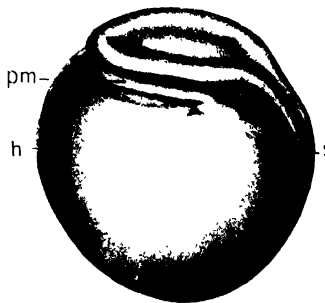Figs. 5–6. Mandibular muscle plate (prechordal mesoderm of earlier stages), lateral plate, and somites in late neurulae of stages 21 and 22; lateral aspect.

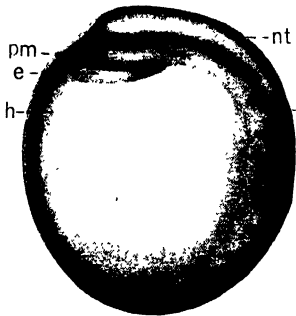### EXPLANATION OF ABBREVIATIONS USED

e., entoderm; *h.*, hypomere; *hp.*, hyomandibular pouch; *mmp.*, mandibular muscle plate; *np.*, neural plate; *nt.*, neural tube; *ov.*, optic vesicle; *pm.*, prechordal mesoderm; *pn.*, pronephros; *s.*, somite.
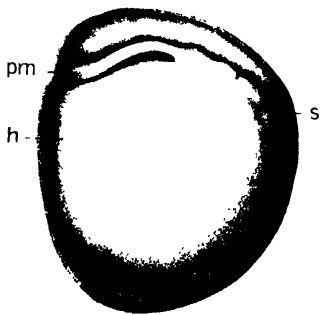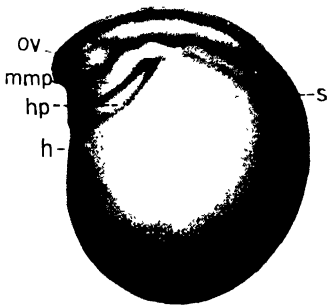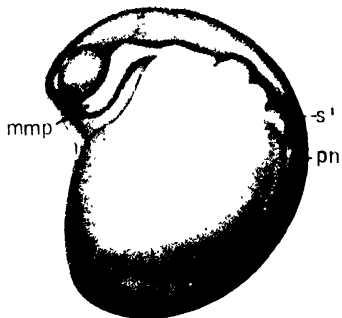
PLATE 9

Differentiation of somites and mandibular muscle plate and development of hyoid and branchial muscle plates in *Triturus torosus* (stages 23–35).

Fig. 7. Mandibular muscle plate, lateral plate, and somites in late neurula of stage 23; lateral aspect.

Fig. 8. Mandibular muscle plate with dorsal proliferation, hyoid muscle plate, and somites in embryo of stage 24; lateral aspect.

Fig. 9. Mandibular, hyoid, and first branchial muscle plates, and somites in embryo of stage 27; lateral aspect. The second, third, and fourth somites show first indications of ventral processes.

Figs. 10–12. Mandibular, hyoid, and branchial muscle plates and somites in embryos of stages 30, 32½, and 35; lateral aspect. The mandibular muscle plate shows first indications of major jaw muscles. In figure 12 the upper end of the hyoid muscle plate has extended to the otic vesicle.

EXPLANATION OF ABBREVIATIONS USED

av., auditory vesicle; *bmp.*, branchial muscle plate; *bp.*, branchial pouches; *h.*, hypomere; *hmp.*, hyoid muscle plate; *hp.*, hyomandibular pouch; *ici.*, intertransversarius capitis inferior; *ics.*, intertransversarius capitis superior; *imp.*, intermandibularis posterior; *lma.*, levator mandibulae anterior; *lme.*, levator mandibulae externus; *mmp.*, mandibular muscle plate; *oe.*, oral evagination; *ol.*, olfactory organ; *p.*, pericardial region; *pn.*, pronephros; *s.*, somite; *t.*, telencephalon; *vp.*, ventral processes of myotomes.
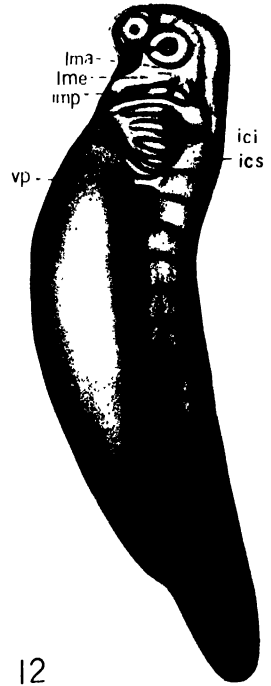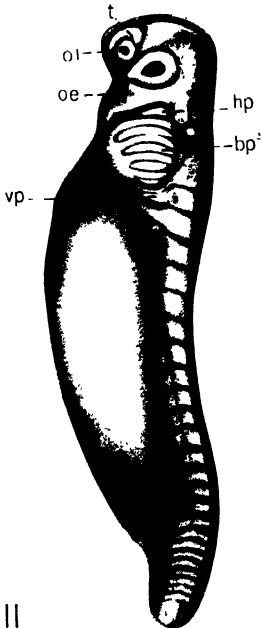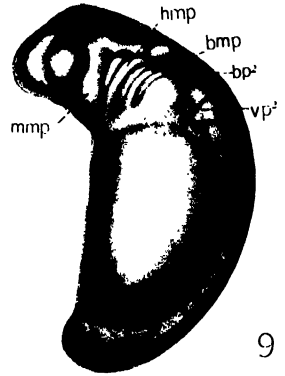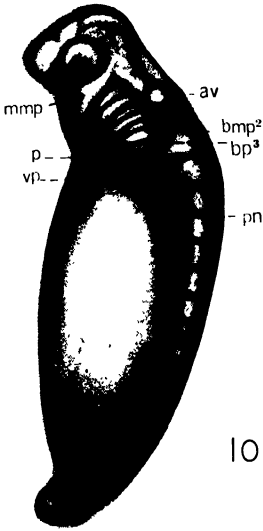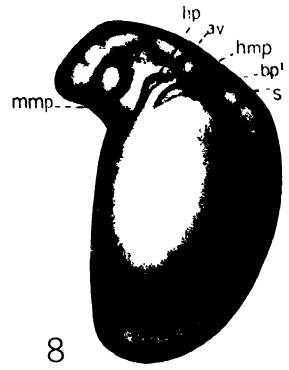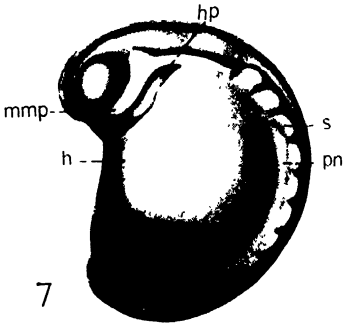
PLATE 10

Muscle rudiments and early muscles of older embryos of *Triturus torosus* (stages 37–39).

Fig. 13. Head muscle rudiments in stage 37; lateral aspect.

Fig. 14. Head muscle rudiments in stage 38½; lateral aspect.

Fig. 15. Early head muscles in stage 39; lateral aspect.

Fig. 16. Early muscles of throat and gular fold on left side of figure; and early hypobranchial muscles on right side of figure, where the more superficial muscle rudiments have been removed; stage 39, ventral aspect.

EXPLANATION OF ABBREVIATIONS USED

av., auditory vesicle; *bhe., branchiohyoideus externus; bm., branchioman-dibularis; dl., dilator laryngis; dm., depressor mandibulae; gh., geniohyoideus; gl.,* glossopharyngeal nerve; *hmp.,* hyoid muscle plate; *ics., intertransversarius capitis superior; ih., interhyoideus; ihp., interhyoideus posterior; imp., inter-mandibularis posterior; l.,* lung bud; *la., levatores arcuum; lma., levator man-dibulae anterior; lme., levator mandibulae externus; oe.,* oral evagination; *ps.,* "premandibular somite"; *rc., rectus cervicis; so., subarcuales obliqui II* and *III; sr I, subarcualis rectus I; sr IV, subarcualis rectus IV; tv., transversus ventralis; v., vagus nerve; vp.,* ventral processes of myotomes.
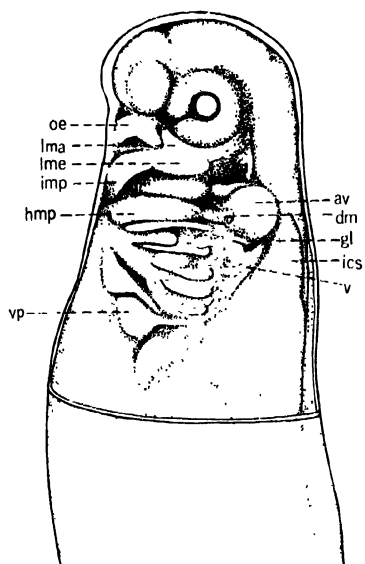
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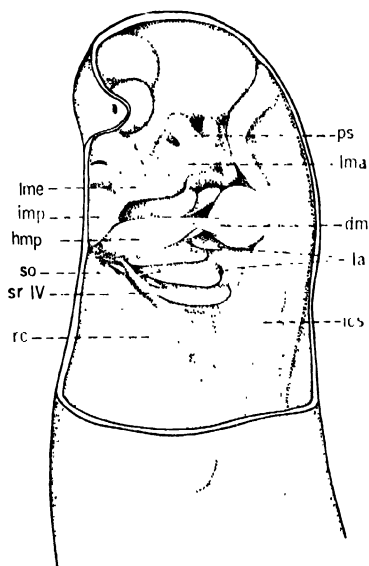
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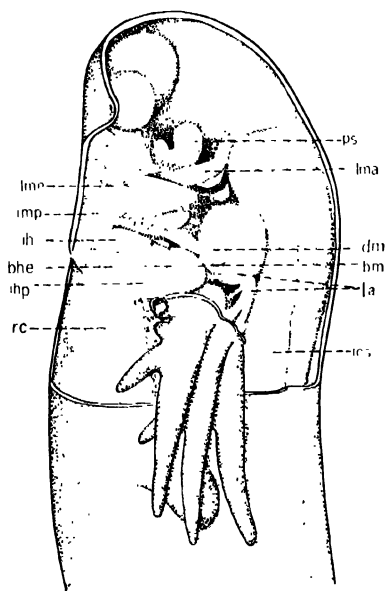
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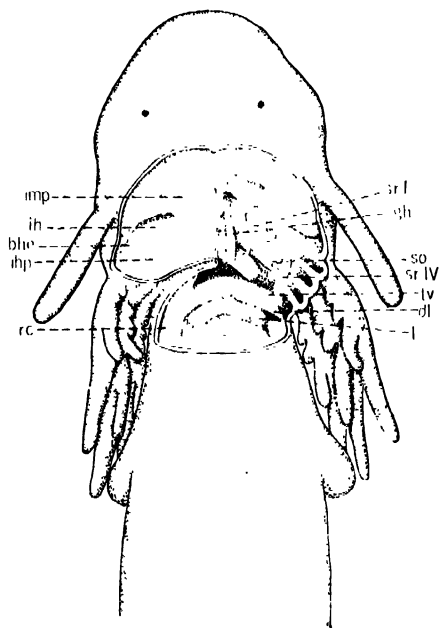
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PLATE 11

Head musculature of young larva of *Triturus torosus* (stage 42).

Fig. 17. Musculature of head; dorsal aspect.

Fig. 18. Musculature of head; lateral aspect.

Fig. 19. Superficial musculature of throat and gular fold, on left side of figure; and hypobranchial musculature, on right side of figure, where the *intermandibularis posterior, interhyoideus,* and *interhyoideus posterior* have been removed with the gular fold; ventral aspect.

Fig. 20. Deeper muscles of branchial region; ventral aspect. A section of the *rectus cervicis* has been removed.

Explanation of Abbreviations Used

bhe., branchiohyoideus externus; bm., branchiomandibularis; db.,depressores branchiarum; dm., depressor mandibulae; dl., dilator laryngis; gh., geniohyoideus; ics., intertransversarius capitis superior; ih., interhyoideus; ihp., interhyoideus posterior; ima., intermandibularis anterior; imp., intermandibularis posterior; la., levatores arcuum; lb., levatores branchiarum; lma., levator mandibulae anterior; lme., levator mandibulae externus; oi., obliquus inferior; os., obliquus superior; re., rectus cervicis; re., rectus externus; ri., rectus internus; rin., rectus inferior; ro., retractor oculi; rs., rectus superior; so., sub arcuales obliqui II and III; sr I, subarcualis rectus I; sr IV, subarcualis rectus IV; tr., transversus ventralis.
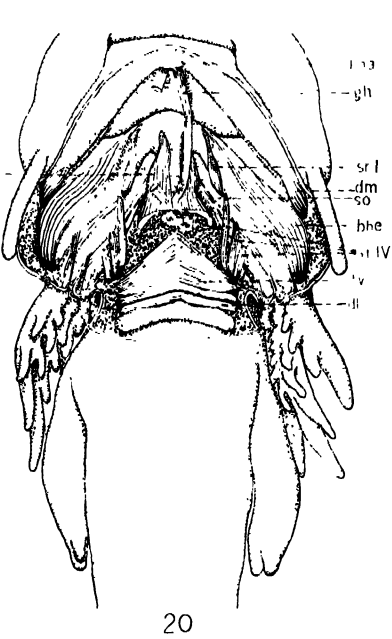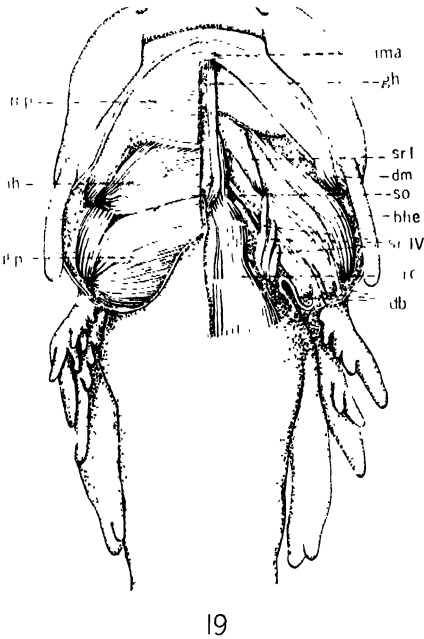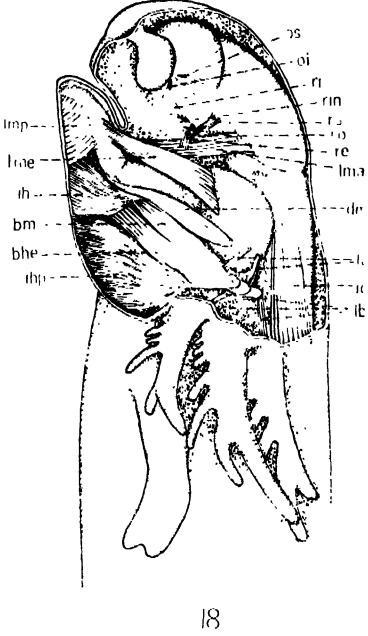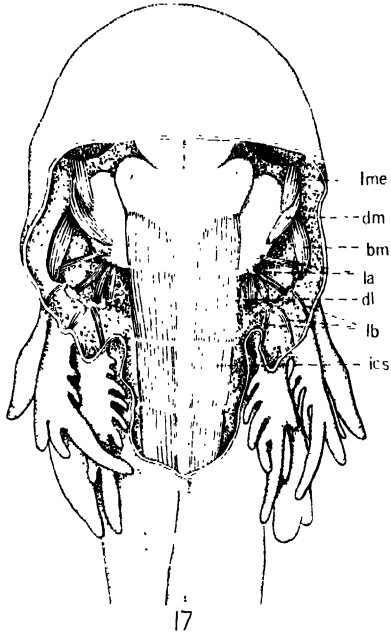
PLATE 12

Head musculature of older larva of *Triturus torosus,* shortly before metamorphosis.

Fig. 21. Musculature of head; dorsal aspect.

Fig. 22. Superficial musculature of head; lateral aspect.

Fig. 23. Deeper muscles of the head, with *levator mandibulae externus* and the *depressor mandibulae* removed; lateral aspect.

Fig. 24. Musculature of throat and gular fold, on left side of figure; and hypobranchial musculature, on right side of figure, where the *intermandibularis posterior, interhyoideus,* and *interhyoideus posterior* have been removed; ventral aspect.

Fig. 25. Deeper muscles of branchial reigon; ventral aspect. A section of the *rectus cervicis* has been removed.

Explanation of Abbreviations Used

bhe., branchiohyoideus externus; bm., branchiomandibularis; c., cucullaris; db., depressores branchiarum; dl., dilator laryngis; dm., depressor mandibulae; gh.,geniohyoideus; ics.,intertransversarius capitis superior; ih., interhyoideus; ihp., interhyoideus posterior; ima., intermandibularis anterior; imp., intermandibularis posterior; la., levatores arcuum; lb., levatores branchiarum; ld., laryngeus dorsalis; lma., levator mandibulae anterior; lme.,levator mandibulae externus; lmp., levator mandibulae posterior; lr., laryngeus ventralis; oi., obliquus inferior; os., obliquus superior; rc., rectus cervicis; rcp., rectus capitis posterior; re., rectus externus; rin., rectus inferior; ri., rectus internus; ro., retractor oculi; rs., rectus superior; so., subarcualis obliquus; sr I, subarcualis rectus; sr IV, subarcualis rectus IV; tv., transversus ventralis.

# THE ORIGIN AND DEVELOPMENT OF THE BLOOD ISLAND OF HYLA REGILLA

BY

ROBERT L. FERNALD

CONTENTS

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INTRODUCTION

THE VASCULAR system was treated as a unit in the first studies of its development; indeed, it was not until the complexity of the factors involved in the morphogenesis of the various elements of the system slowly became apparent that the method of approach was changed. Prior to 1915 most studies on the development of the circulatory system in lower vertebrates (Schwink, 1891; Brachet, 1898, 1903, 1921; Rückert and Mollier, 1906; Maximow, 1910; Hilton, 1913) were largely descriptive in nature and sought to establish the derivation of the various vascular tissues in accordance with the principle of specificity of germ layers. Agreement on this aspect of the problem has not yet been attained.

With specialization of interest and the use of the experimental method, certain phases of the general problem have received more attention; for example, our knowledge of the development of the amphibian heart has been greatly extended by the researches of Ekman (1925), Stöhr (1924, 1927), and Copenhaver (1926, 1939). The anlage of the blood island of amphibians, on the other hand, while much more diffuse and extensive than that of the heart, constitutes a very compact structure when compared with the early blood-forming areas of the embryos of other vertebrates. Experimental studies on the origin of hemopoietic tissue in amphibians have been made by Federici (1926), Goss (1928), Slonimski (1931), Stöhr (1931), and Storti (1935).

The term "blood island," first introduced according to von Baer (1828, p. 31) by Caspar Wolff as descriptive of the scattered masses of developing blood cells in the area vasculosa of the chick embryo, has been adopted to apply to the single, "compact" anlage of the blood in amphibians. This usage of the term is not to be confused with the original meaning nor yet with the many interpretations given it when applied to the early blood-forming centers in other vertebrate groups.

The present study, in addition to including an examination of the features of normal development of the blood island of *Hyla regilla*, attempts: first, to determine the source and degree of determination of the presumptive blood island cells in the earliest stages of gastrulation; second, to ascertain by extirpation of these cells at later stages of development what cellular elements of the larval blood are derived from them; third, to determine, if possible, whether other sources of bood cells exist in the embryo; and, fourth, to test the effect of inducing tissues like the chorda upon the course of development of the cells of the blood island.

It is a pleasure to acknowledge the assistance and helpful criticism of Professor J. Frank Daniel,[*] under whose direction this problem was undertaken. I wish also to acknowledge the generous assistance of Dr. Richard M. Eakin.

MATERIALS AND METHODS

Embryos of the Pacific Coast tree frog, *Hyla regilla* (Baird and Girard), in various stages of development, were used. This small tree frog, the most abundant and easily available anuran type in the San Francisco Bay area, has an extraordinarily

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[*] Since deceased.

long spawning season, which under favorable climatic conditions may extend from early January to late June. The embryos develop rapidly, reaching metamorphosis in 60 to 80 days.

The resistance to infection exhibited by embryos of *Hyla* makes complete asepsis in operating unnecessary. Sterile salt solution, prepared by diluting Holtfreter's solution (Holtfreter, 1931) by one half with distilled water, was used as an operating medium. Operations were performed with microknives (Burch, 1938) and fine iridectomy scissors. Experimental and control animals were cultured in 50-per cent Holtfreter's solution in Stender dishes or in open finger bowls. A piece of cellophane on the bottom of the culture dishes prevented the embryos from sticking to the glass

Fig. 1. Diagram to show the distribution of materials in the early anuran gastrula. Arrow is pointing toward blood island cells. From Holtfreter (1938).

surface. The solutions were changed daily. After two days the salt solution was replaced by pond water. Larvae were fed small amounts of Heinz's strained spinach daily.

Young stages, in which yolk was still abundant, were fixed in Smith's fluid; all other embryos, in Bouin's fixative. Paraffin sections were prepared and stained with Harris' haematoxylin and either eosin or triosin. For study of the normal development of the blood island, serial sections of embryos at various stages of development were prepared. Slonimski's (1927) modification of Wu's benzidine technique was used to demonstrate grossly the distribution of tissues containing hemoglobin.

NORMAL DEVELOPMENT OF THE BLOOD ISLAND

It is from the marginal zone of the early gastrula that the primary mesoderm of the later embryo is derived. The blood island is developed from cells in the segment of this zone opposite the blastopore (fig. 1). Since these cells lie in the inner part of the marginal zone facing the segmentation cavity, it is impossible to stain them with vital dyes.

The marginal and vegetal zones are carried inside the embryo and are covered by the cells from the animal zone in the processes of gastrulation. These processes so distribute materials that a three-layered condition is achieved before neurulation is started. When the blastopore is slit-shaped, the mesoderm for the notochord,

somites, and the lateral plates forms a continuous layer between the ectoderm and entoderm in the posterior half of the embryo; the dorsal mesoderm, however, extends farther anteriorly. The cells which are to contribute to development of the blood island now form a part of this mesodermal layer and are scattered along the venter from the ventral lip of the blastopore anteriorly into the free edges of the lateral plates in the region of the liver diverticulum.

During neurulation there is little change in the appearance of this part of the mesoderm. At the stage of development characterized by the closing of the medullary folds, the ventral mesoderm becomes closely applied to the entoderm. Separation of the two layers, which is easy prior to this stage, becomes almost impossible. As the embryo progresses to the tail-bud stage, the ventral mesoderm again becomes separate. This secondary fusion of the germ layers was reported by Mietens (1909) in *Bufo*, and, as he suggested, might well account for much of the confusion concerning the origin of the cells of the blood island.

By the time the embryo (at about 5 mm.) first responds to stimuli, definite cellular differentiation of the blood island has taken place. The formation of the island in *Hyla regilla* is not preceded by the separation of a thick plate of cells from the ventral mesoderm, as has been reported for many other forms by Hilton (1913), Brachet (1921), Goda (1927), *et al.* Cells are proliferated from the side of the thin layer of ventral mesoderm toward the entoderm, and form a loose mass. The extent of the blood island at this stage is clearly demonstrated by Slonimski's (1927) benzidine technique, which selectively stains tissues containing hemoglobin a bluish black (fig. 2). The island extends along the midventral line from the anus to the liver, bifurcates, and continues to both sides of that organ. About twenty-four hours later when the heart starts to pulsate these anterior extensions become connected with it.

The cells of the blood island in the 5 mm. stage lie in grooves in the surface of the yolk mass and are separated by developing endothelial cells from the much larger yolk-laden cells. With the completion of the development of the endothelium, a central vessel, later the subintestinal vein, is established with many lateral branches; the whole complex is called the vitelline system.

Fig. 2. Diagrammatic ventral view of a 5-mm. larva. The blood island is stained by the benzidine technique.

When in a 5-day-old embryo the blood cells are released into the circulation, they carry yolk platelets, and the blood is colorless or milky in appearance. The erythrocytes of the 8-day-old larva with the operculum covering the gills still carry yolk platelets, but the blood has become pink in color. It is possible to identify the smaller white cells at this stage, but not earlier, when the cells are so packed with yolk platelets as to conceal the white cells. In the feeding stage, the blood is red, and yolk platelets are absent.

Throughout the larval period, mitoses of the blood cells in the circulating medium are frequently found. This repeated division of corpuscles derived from the blood island is the only apparent source of the increasing number of erythrocytes during the first 15 days of development. The mesonephroi and the associated hematopoietic tissue begin to develop in larvae at about 16 days, a stage characterized by the appearance of the hind limb buds. The spleen does not become active as a hemopoietic center until two days later.
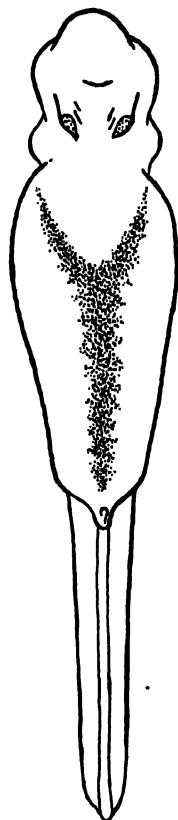
EXPERIMENTAL WORK

Explantation of Early Gastrular Material

Slonimski (1931) and Storti (1935) established the fact that the blood island from the neurula was capable of self-differentiation into erythrocytes. Holtfreter (1938) explanted presumptive blood island cells together with such associated material of the early gastrula and obtained differentiation of erythrocytes. The series of explants to be described here includes a smaller number of cells from a more restricted region than Holtfreter used.

Embryos in the first stages of gastrulation, that is, when the blastoporal groove was just indicated, were used as donors. A small block of cells was excised from the marginal zone at a point directly opposite the blastoporal groove (fig. 3) and enclosed within a small sheet of presumptive epidermis cut from the ectoderm overlying the segmentation cavity in embryos of the mid-gastrula or large yolk-plug stage. A control series of explants consisting of this presumptive epidermis alone showed the development of epidermis only. Such explants do not form vesicles; rather, they form small wrinkled masses of unorganized epidermal cells. The production of vesicles occurs only in the presence of mesodermal cells.

Of 71 explants of material from the ventral marginal zone, 58 were maintained for 8 days, fixed in Bouin's, and sectioned at 8μ. Differentiation of blood cells was characteristic of the series. A typical explant (see pl. 1, fig. *a*) is, in addition to the outer epidermis and subjacent layer of mesenchyme, composed only of blood cells (*bc.*). Many of the erythrocytes have lost the yolk platelets and have assumed the ovoid character of larval blood cells. Epithelium, characteristic of the intestinal tract, is present in small amounts in a few of the other explants.

Fig. 3. Diagram of sagittal section of the early gastrula. *bp.*, blastopore; stippled area, presumptive blood island.

This experiment demonstrates that at the time of appearance of the blastoporal groove, cells in the ventral part of the marginal zone are capable of self-differentiation into red blood cells; hence, they possess at least labile determination for their presumptive fate.

Extirpation of the Presumptive and Definitive Blood Island

Defect experiments have been a popular and instructive procedure of the experimental embryologist since Roux first introduced them. In the study of the development of the blood island, this method has been applied by several workers (Federici, 1926; Goss, 1928; Slonimski, 1931; Stöhr, 1931; Storti, 1935). In the present study complete ablation of the presumptive or definitive blood island was attempted on 840 embryos, namely, 240 in the slit-shaped blastopore stage, 90 neurulae, and 510 in tail-bud and young larval stages.

Operations on preneurula and neurula stages were limited to extirpation of the sheet of ectoderm and mesoderm from the ventral lip of the blastopore forward to the region of the thin floor of the foregut and halfway up on both sides, leaving a large area of entoderm exposed. This drastic operation resulted in high mortality. In the later stages, where the exact distribution of the blood island could be demonstrated by the benzidine technique (see fig. 2, p. 131), fine iridectomy scissors were used to excise the whole region, including the underlying entoderm. Here again fatalities were numerous as a result of loss of yolk, incomplete healing, or injury to

the pronephric region. Attempts to aid healing and recovery by covering the exposed entoderm with a sheet of epidermis complicated the results. The fusion of the yolk to the epidermal covering resulted in marked abnormalities of the digestive tract and, eventually, in death.

There was a marked reduction in the number of blood cells in the animals in this series, many of which showed only an occasional cell in the fluid circulating through the gills. However, no embryo maintained for the few days necessary for recovery and the establishment of circulation was completely deprived of the cellular elements of the blood, either red or white.

Embryos which survived the operation and did not lose so much yolk as to prevent the establishment of a complete digestive tract, were easy to maintain as long as desired, notwithstanding the reduction in blood cells. The gills of these embryos were slower to develop and smaller than normal. The operculum was likewise slow to cover the gill area. The heart and associated vessels developed in a normal fashion, except when the connection between veins and heart was prevented from forming, or when the embryo became edematous as a result of injury to the pronephroi. In the latter instances the heart remained tubular and almost straight. Daily examination showed a gradual increase or regeneration of the cellular components of the blood; by the end of the third week the blood had taken on a red color owing to the large number of erythrocytes.

These gross observations on living embryos were confirmed by serial sections of animals fixed at intervals after the operation. Blood cells, both red and white, were present in reduced numbers in embryos fixed in early stages. The report of Slonimski (1931) that the white cells were present in relatively greater percentages than in the normal animal could not be confirmed for *Hyla*, except in a few embryos in which the healing was slow or incomplete.

Notwithstanding almost complete elimination of the cellular components of the blood, development of the blood vessels was relatively unaffected; the endothelium of the major vessels developed in spite of the ablation of the island. This result is in agreement with Cameron's (1940) observations on X-rayed neurulae of *Amblystoma jeffersonianum*. Cells which develop into endothelium and those which form red blood cells were shown to have a different susceptibility to X-rays. This indicates a distinct physicochemical difference in the two types of cells and a possible difference in their origin.

Blood cells in mitosis were found in the circulating blood 10 days after operation in relatively larger numbers than in the normal larvae. Repeated division was the only apparent method of increasing the number of erythrocytes. There was no indication that the endocardium and myocardium were at any time erythropoietic, a condition described by Ichikawa (1934) in a study of compensatory hemopoiesis in *Hynobius retardatus*. In experimental animals fixed 18 days after the extirpation of the blood island, hemopoietic activity was pronounced in the mesenteries, spleen, and mesonephroi. By this time the blood possesses a definite red color and large masses of blood cells are present in the lumina of the heart and the larger blood vessels. This rapid compensation for the loss of most of the early hemopoietic tissue stands in contrast to the findings of Federici (1926) and Slonimski (1931) that blood cells fail to regenerate in animals almost deprived of derivatives of the blood island.

The results of this series of operations agree in one essential with those of other workers who have extirpated the blood island, namely, in that the blood island is at least the major, if not the only, source of erythrocytes in the early developing amphibian. Federici (1926), Goss (1928), and Stöhr (1931) have reported com-

plete elimination of cells from the blood by ablation of the blood island. "Bloodless" embryos obtained by Federici were fixed 6½ days after the operation, and he admits that "Par ci par là, sur certaines coupes, on peut y voir une cellule douteuse" (p. 476). The presence of doubtful cells in embryos sacrificed so early in development and the occurrence of a small number of erythrocytes in all animals allowed to develop longer detract from the force of his report. More authentic examples of the production of embryos without erythrocytes are those of Goss and Stöhr. The former obtained 4 larvae of *Amblystoma punctatum* without blood cells from a series of 30 operations; the oldest was maintained for 32 days. Stöhr found 13 embryos with no blood cells from a series of 320 experimental animals; these were fixed 10 to 20 days after the operation.

CEPHALIC AND CAUDAL MEROPLASTS

Embryos which had developed to the stage of first response to stimulus were transected just posterior to the developing heart (fig. 4). In order that a complete heart might develop and establish connection with the anterior veins, the cut was made close to the anterior extensions of the blood island. Since the limits of that structure are not easily distinguished externally, the accuracy of the cut could not be assured. The purpose of this simple operation was to obtain an anterior part without the blood island and a posterior part containing the complete blood anlage. The two pieces of the embryo were cultured separately in salt solution and fixed after 10 days.
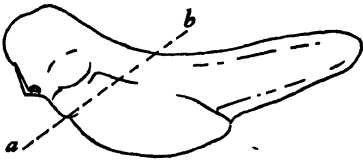
Fig. 4. Diagram of a 5-mm. larva. Line *ab.* shows position of transection in the meroplastic series.

The cut surface of the anterior part closed over slowly and in about 75 per cent of the experiments healed completely after 24 hours. The development of these meroplasts closely paralleled that of the anterior end of a complete embryo. The heart became functional in most instances; gills developed and were covered by the opercular folds; the movement of the jaws in the feeding stage was characteristic of that in normal larvae. It was possible to maintain for 10 days, only 17 out of a series of 30 meroplasts.

A careful examination of serial sections of these cranial fragments showed the presence of all structures typical of the anterior part of the body with the exception that in four specimens erythrocytes were completely absent. In three of these no trace of other cellular elements of the blood was found. The fourth specimen had three "doubtful" cells. An example typical of the first three may be described as follows: Differentiation of tissues is well advanced. Yolk platelets have disappeared from all cells. The lumen of the heart is open and the heart had been functional; that is, circulation had been established. Blood vessels are well developed. Pronephric tubules are present on both sides. Stomach and anterior segment of the duodenum with masses of pancreatic and liver tissue are clearly differentiated. Gills are reduced in size. The brain and sense organs are normal. No blood cells of any kind are present. The complete development and normal differentiation of all tissues of this cephalic meroplast, with the exception of cellular components of the blood, should be emphasized.

The development of a complete and functional system of blood vessels directly associated with the heart in this cephalic fragment demonstrates the ability of endothelial tissue to develop independently of the blood island. The complete absence of blood cells is of interest as hemogenic activity of endothelium has been

reported for various classes of vertebrates by several workers: Ichikawa (1934), Jordan (1916), Reagan (1915, 1917), Sabin (1917, 1920, and 1922), Storti (1931*a*, *b*), and others.

In a study of embryos of *Fundulus heteroclitus,* chemically treated to prevent the circulation of the blood, Stockard (1915) found no evidence of the production of red blood cells from the endothelium. Reagan (1915, 1917), in an examination of similarly treated *Fundulus* embryos, reached the opposite conclusion—namely, that the endothelium is capable of transforming into blood cells. R. D. Lillie (1919) described the formation of large lymphocytes capable of producing all types of white blood cells, from the endothelium of developing *Bufo halophilus.* Storti (1931*a*) attributed hemogenic activity to all the endothelium of the amphibian larva until the time of metamorphosis. Indeed, this capacity of the endothelium for hemopoiesis was regained in animals splenectomized after metamorphosis (Storti, 1931*b*, 1932). Ichikawa (1934) and Jordan and Speidel (1930) limited blood-forming activity of endothelium to that of the heart. Federici (1926), Goss (1928), and Slonimski (1931) did not attribute a capacity for erythropoiesis to the endothelium of early larvae. Stöhr (1931) concluded that the endothelium of the heart of very young larvae of *Bombinator* "very probably" was capable of forming blood cells. This ability, he thought, was lost early in development.

The absence of both red and white blood cells in the cephalic meroplasts of *Hyla* clearly indicates that under the conditions described the endothelium of the anterior vessels and heart does not possess the capacity for hemopoesis. The normal development and differentiation of all other tissues and the presence of a circulating fluid in the heart and vessels combine to make a situation closer to the normal than that in the chemically treated embryos of *Fundulus* studied by Reagan (1915, 1917) and Stockard (1915). The absence of circulation and the presence of abnormalities in other tissues characterized their chemically treated animals.

The cut surfaces of the posterior meroplast healed more rapidly than those of the cephalic meroplasts. Again, development closely paralleled that of the posterior end of a normal embryo, except that it progressed more slowly. The headless embryos became motile and responded more violently to stimuli than did normal larvae. As was to be expected, these caudal meroplasts were "albinos."

The blood island differentiated into a large mass of corpuscles within the vitelline vessel and became red in color. The thin vessel containing the red mass of erythrocytes branched, and in some instances the color spread with the branching. More often the corpuscles remained localized along the developing gut. Examination of these embryos in sections shows them to be normal except that the gut has differentiated more slowly than in complete animals. The "red masses" are composed of both red and white corpuscles. The erythrocytes are free of yolk platelets and have the typical ovoid shape. Although these caudal fragments have differentiated far beyond the "hatching" stage, there is no evidence of a disintegration of the products from the blood island with subsequent replacement by larger erythrocytes from "other haemopoietic" centers, as described by Cameron (1941) in *Amblystoma jeffersonianum.*

EXPLANTATION OF PRESUMPTIVE BLOOD ISLAND AND PRONEPHROS SINGLY AND WITH ORGANIZER MATERIAL

The role of the organizer in the determination of the trunk mesoderm of *Triturus* spp. has been investigated by Yamada (1937, 1940), who reports that the differentiation of the trunk mesoderm of the early neurula is, within certain limits, dependent upon its position with reference to the organizer. For example, pre-

sumptive blood island cells brought into close contact with the dorsal organizer form, in part, pronephric tissue; likewise, presumptive pronephric material removed from the influence of the organizer is capable of forming the tissues typically derived from more ventral mesoderm, namely, erythrocytes and body wall. To determine whether the organizer exercised a similar effect on the trunk mesoderm of *Hyla regilla,* four series of explants were made.

The general procedure was the same as in explantation of gastrular material. Early neurulae in which the medullary plate was developed sufficiently to make certain the identification of areas on the embryo served as donors in all instances. The cells to be explanted were rolled in a sheet of presumptive epidermis cut from the ectoderm overlying the segmentation cavity in embryos of the mid-gastrula or large yolk-plug stage. Eight days were sufficient for complete differentiation of the explanted cells. At the end of this time, the explants were fixed in Bouin's and sectioned at 8 μ. The mortality rate was high in all series and especially so in the series in which neural and chordal tissues were present. About one-sixth of the explants survived for 8 days.

EXPLANTS OF THE PRESUMPTIVE BLOOD ISLAND

The strip of ectoderm and mesoderm along the midventral line from below the blastopore forward one-third the length of the embryo was excised (fig. 5) and rolled in a sheet of presumptive epidermis. The healing of the epidermal bag about the explant required less than an hour. After two days the explant became vesicular and moved about by means of the ciliated epidermis. In most instances a small ventral fin and proctodeum were developed from the ectoderm taken from the region ventral to the blastopore.

Sections of the explants showed the wall of the vesicle to be composed of epidermis and underlying mesenchyme. The central cavity was lined by a layer of flattened cells. In 24 of the 27 explants fixed for study, developing blood cells were present. Two of the remaining explants were devoid of blood cells, a third had a mass or conglomerate of cells at one side of the central cavity which was not identifiable. One explant contained, in addition to blood cells, a small pronephric tubule; another developed a few muscle fibers in the wall of the isolate. The degree of differentiation of the blood cells was comparable to that in the explants of gastrular material (pl. 1, fig. *a*).

COMPOUND EXPLANTS OF PRESUMPTIVE BLOOD ISLAND AND
NEUROCHORDAL MATERIAL

This series of explants differs from the one just described in that neural and chordal material from the early neurula was included (fig. 6). A control series of neural and chordal material cultured alone showed these cells to be capable of differentiating into neural, chordal, and skeletal muscular tissues, but not into blood cells or pronephric tubules. The muscle tissue becomes functional after 4 days, as may be demonstrated by a spasmodic twitching of the explant when stroked gently with a hair loop.

The number of explants, out of a total of 29 for the series, showing the formation of different structures is summarized as follows: 29, neural tissue; 29, notochord; 26, muscle; 22; pronephric tubules (1 doubtful explant) ; 10, blood cells (5 doubtful explants). The differentiation of pronephric tubules, characteristic of 76 per cent of the explants, indicates an inducing effect of the neurochordal material on the mesoderm of the presumptive blood island. Renal structures in these explants are shown in plate 1, figures *b* and *c*.

EXPLANTS OF PRESUMPTIVE PRONEPHROS

The material which forms the pronephros in the normal development of an early neurula is located in the layer of mesoderm lying beneath the ectoderm lateral to the medullary folds. This sector of mesoderm (fig. 7) with its overlying ectoderm was explanted and cultured in an epidermal bag. Thirty-one specimens were maintained for the 8 days necessary for differentiation. Of these explants only 3 contained both pronephric tubules and blood cells; whereas 26 were characterized by the presence of blood cells alone. Skeletal muscle was also found in 8 of the vesicles.
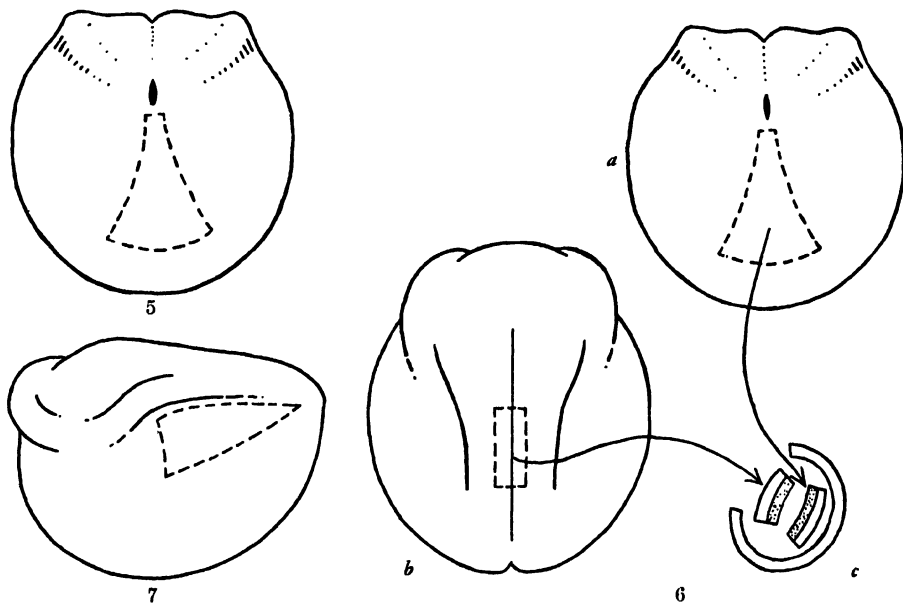
Fig. 5. Diagram of the posterior end of an early neurula to show the source of material explanted as presumptive blood island.

Fig. 6. Scheme for the preparation of compound explants of presumptive blood island and neurochordal material from the early neurula. *a*, posterior view of the donor of presumptive blood island; *b*, dorsal view of the donor of neurochordal material; *c*, compound explant rolled in presumptive epidermis from an embryo in mid-gastrula stage.

Fig. 7. Diagram of an early neurula to show the source of material explanted as presumptive pronephros.

COMPOUND EXPLANTS OF PRESUMPTIVE PRONEPHROS AND
NEUROCHORDAL MATERIAL

A small block of neural and chordal material cut from the early neurula (fig. 6), and presumptive pronephros excised as described for the preceding series, were cultured together in an epidermal bag. Twenty-two such explants were prepared for histologic examination.

Each explant exhibited neural, notochordal, skeletal muscular, and pronephric structures. The pronephric tubules were usually closely associated with the notochord, but there were exceptions (pl. 1, fig. *d*). The differentiation of other tissues characteristic of this series is illustrated in plate 1, figure *e*. Three of the explants contained a few doubtful cells suspected of being blood cells. One explant had developed a small mass of tissue, almost cut off from the main vesicle, which con-

tained a few well-differentiated blood cells. The results of these four series of explants are summarized in table 1.

A comparison of the results obtained by explanting presumptive blood island and presumptive pronephric material of the early neurula shows that the cells from the two regions tend to differentiate similarly; that is, to form tissues typical of the ventral mesoderm. Furthermore, the trend of differentiation of these two materials when explanted with cells of the organizer region is similar. There is a marked tendency to develop tissues characteristically formed from the lateral mesoderm close to the organizer center. The response of the ventral mesoderm or presumptive blood island to the organizer is less complete, however, than that manifested by the presumptive pronephros.

TABLE 1

SUMMARY OF RESULTS OF EXPLANTATION

Series and type of explant	No. of explants	Percentage of explants containing				
		Neural tube	Notochord	Muscle	Pronephros	Blood cells
1. Presumptive blood island.	27	0	0	4–	4–	89.0
2. Presumptive blood island and neurochordal material...................	29	100	100	89	76	37.0
3. Presumptive pronephros..	31	0	0	26	10	93.5
4. Presumptive pronephros and neurochordal material...................	22	100	100	100	100	4.5

The activity of the presumptive blood island in explants indicates only a labile determination of that material, which may be modified in most instances by the presence of neurochordal material. The capacity of the presumptive pronephros to differentiate blood cells demonstrates the extent of the potential blood-forming tissue in the early neurula.

These results lend support to the hypothesis of a mechanism of determination in the trunk mesoderm of *Triturus*, elaborated by Yamada (1940). Yamada declares the various divisions of the trunk mesoderm are to be characterized by a certain capacity for differentiation, a "morphogenetic potential." The morphogenetic potential of the ventral mesoderm of an early neurula is of a low value and, when tested by isolation of the material, is expressed in the differentiation of blood cells. At this stage the lateral mesoderm which later forms pronephros has a morphogenetic potential comparable to that in the ventral mesoderm, and in explants in most instances forms blood cells. In these early stages the morphogenetic potential of a part of the mesoderm is subject to change, which normally is induced by the action of the organizer. Yamada states that the potential of the ventral mesoderm, cultured with neurochordal material, may be raised by the action of the organizer to a higher threshold which causes it to differentiate not as blood cells but as pronephric tubules. This does not occur in the normal embryo because the ventral mesoderm is beyond the field of action of the dorsal organizer. The presumptive pronephric material does lie within the field of the organizer, and its morphogenetic potential is raised during the course of neurulation to the threshold necessary for the differentiation of pronephros.

Yamada's theory, thus briefly outlined, offers an explanation of the effect of the neurochordal material on the presumptive blood island in explants, and also indi-

cates a possible explanation for the development of blood in the embryos from which the presumptive blood island was extirpated in preneurula and neurula stages. The most dorsal part of the lateral mesoderm, in growing down with the overlying ectoderm to cover the entoderm exposed by the extirpation, would, according to this hypothesis, leave the field of action of the organizer; hence, the value of its morphogenetic potential would remain low and differentiation of blood cells would result. Yamada (1937) transplanted presumptive pronephros, stained with Nile-blue sulphate, to the ventral region of early *Triturus* neurulae, and reported the formation of blood from the stained material. Interesting as such an experiment on *Hyla* would be, the difficulty of adequately staining the mesoderm in anurans prevents its application.

TRANSPLANTATION OF ORGANIZER MATERIAL TO THE REGION OF THE PRESUMPTIVE BLOOD ISLAND

A strip of medullary plate and underlying chordamesoderm was excised from the mid-dorsal region of the early neurula. The strip, which included material from a point just anterior to the anlagen of the optic vesicles to the posterior end of the developing medullary plate, was transplanted to the midventral region of a second embryo in the early neurula stage. This animal, the host, was prepared to receive the graft by making an inverted T-shaped incision in the ventral ectoderm and mesoderm: the bar of the T was made a short distance anterior to the region in which the proctodeum was to form, and the standard of the T extended forward along the midventral line to the liver region (fig. 8). The cut edges of ectoderm and meso-

Fig. 8. Scheme for the preparation of the transplantation series: *a*, dorsal view of the donor of the strip of medullary plate and underlying chorda and somite mesoderm; *b*, ventral view of host prepared to receive the graft.

derm were gently rolled back, the transplant was placed upon the entoderm in normal anteroposterior orientation, and the cut edges of graft and host were brought together with a hair loop. To facilitate healing the graft was held in place for about 45 minutes with a small glass bridge.

One control group of embryos was subjected to the same operative procedure with the exception that a strip of presumptive epidermis was substituted for the neurochordal material of the graft. A second control series, which more nearly simulated the strain and tension in the developing graft, was devised by preparing two early neurulae as though for hosts in the experimental series and fusing the cut surfaces together; thus, parabiotic twins, fused venter to venter, were obtained. Healing in all animals was complete after 12 hours.

Two sets of experimental animals were prepared: one set was sacrificed after 6 days; the second, after 7 days. The control series of the first type was prepared at the same time from the eggs of the same mass as the first set of experimental animals. The parabiotic controls were made from eggs of the same mass at the time of preparation of the second set of experimental animals. All the embryos were cultured under similar laboratory conditions. ·

The embryos bearing the graft of medullary plate and chordal material, developed in an apparently normal manner except for a reduction in the size of the

external gills. The transplant completed neurulation, and small abnormal optic vesicles were developed at the anterior end of the graft. The graft in most instances extended from the posterior region of the pericardial cavity to the anus. The development of the anal opening was complete and thus edema was avoided. The graft exhibited twitching movements, independent of the activity of the host.

Serial sections were prepared and the blood cells counted. A comparison of the value obtained by counting the blood cells present in each section with a multiple of ten of that value (obtained by counting those in every tenth section), showed a variation of less than 5 per cent, the complete count always being less than the incomplete. Table 2 lists such counts in every tenth section. These figures represent of course only one-tenth the total number of blood cells and are not exact cell counts; rather, they represent very close approximations.

TABLE 2

Counts of Blood Cells in Controls and in Series of Transplantations

Six-day experimental series		Six-day control series		Seven-day experimental series		Seven-day parabiotic control series	
Animal	No. of cells	Animal	No. of cells	Animal	No. of cells	Animal	No. of cells divided by 2
32	418	1	1,280	43	158	1	2,157
34	191	2	957	44	525	2	2,028
35	70	3	1,892	45	369	3	2,479
36	74	4	1,305	46	398	4	2,241
37	487	5	1,432	47	158	5	2,393
38	160	6	1,325	48	233	6	2,121
40	73	7	1,276	49	459	7	2,382
41	81	..	.....	50	982	..	.....
Avg. 194		Avg. 1,352		Avg. 410		Avg. 2,257	

The marked reduction of the number of cells present in the blood stream of both experimental groups is apparent. The six-day series contained only 14 per cent of the number present in the control group; the seven-day series, only 18 per cent. There was a complete absence of pronephric tubules in the tissues which differentiated in and around the graft, but neural tube, notochord, somites, and primitive germ cells were clearly differentiated. The anterior end of the graft had developed parts of the brain and optic cups. A large blood vessel was usually present in the body wall on each side of the graft. In one instance, number 35, a small isolated pocket of endothelium filled with blood cells was located at the side of the graft. In most specimens the mesenchyme of the graft and adjacent tissues of the host contained a greater number of cells, undifferentiated, than in the normal animal. In some instances, cells similar to these were present in the mesodermal layers of the intestinal wall. The graft in embryo 50 of the seven-day series was far back on the trunk and to one side; this individual is therefore not comparable to the other members of that series.

Because the number of experimental and control animals was small, definite conclusions cannot be drawn. A marked reduction in number of blood cells in the experimental animals, however, is clear. To determine the factors responsible for this reduction requires further experimentation. In these few instances there has been no indication of the induction of the ventral mesoderm by the organizer mate-

rial to cause its differentiation into pronephric tubules in place of blood cells. It is possible that the rapidly growing and differentiating graft is not conducive to blood formation. Danchakoff (1916) has stated that the absence of rapidly growing structures and of contracting muscles is a necessary condition for the formation of blood in both embryo and adult. However, in a few specimens of the extirpation series on which the operation had not been complete, groups of cells from the presumptive blood island were observed to be carried back by the growing tail bud and thus isolated. These isolated masses were found in sections to be differentiated into blood cells in spite of having been surrounded by striated muscle of the tail. In these instances the developing blood cells were more closely associated with rapidly developing, contractile tissue than in the transplants. This evidence is too limited to be considered as more than an indication that cells from the presumptive blood island can differentiate under conditions considered to be unfavorable to their development.

SUMMARY AND CONCLUSIONS

1. The blood island of *Hyla regilla* is a derivative of the ventral mesoderm. Both axial and peristomial mesoderm contribute to its formation.

2. Cells from the presumptive blood island in the marginal zone directly opposite the developing blastoporal groove of the early gastrula, when cultured in explants, possess the capacity for self-differentiation into blood cells.

3. Extirpation of the blood island in the slit-shaped blastopore, neurula, and tail-bud stages result in marked reduction of the number of blood cells. No animals were obtained entirely without blood cells.

4. Head meroplasts with no derivatives of the blood island but with a functional circulatory system develop without either red or white blood cells. Under these conditions the endothelium of the heart and anterior vessels is not capable of hemogenic activity.

5. The development of endothelium may be independent of the blood island, as is indicated by the establishment of a complete system of vessels following extirpation of the island and in head meroplasts.

6. Explants of presumptive blood island and pronephros of the early neurula show the differentiation of blood cells. The extent of potential blood-forming tissue is indicated. At this stage not only ventral mesoderm, but also the mesoderm of the presumptive pronephros and adjoining lateral plate, is capable of developing into blood cells. This offers a probable explanation for the development of blood cells in embryos from which the blood island was extirpated in the neurula stage.

7. Presumptive blood island and pronephros of the early neurula explanted with neural and chordal material produced pronephric tubules in a high percentge of explants. The role of the dorsal organizer in the determination of trunk mesoderm is considered.

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PLATE 13

a. Section of an explant of presumptive blood island from the early gastrula showing blood cells, *bc* and endothelium, *c*.

b. Section of an explant of presumptive blood island and neurochordal material showing pronephric tubules, *p*, associated with striated muscle, *m*.

c. Section of an explant of presumptive blood island and neurochordal material showing a pronephric tubule, *p*, associated with the notochord, *ch*; *m*, muscle; *n*, neural tissue.

d. Section of an explant of presumptive pronephros and neurochordal material. *m*, muscle; *p*, pronephros.

e. Section of an explant of presumptive pronephros and neurochordal material showing the degree of differentiation of notochord, *ch*; striated muscle, *m*; and neural tissue, *n*.

FERTILIZATION OF COELOMIC EGGS OF TRITURUS TOROSUS

BY

G. MERLIN GOOD AND J. FRANK DANIEL

FERTILIZATION OF COELOMIC EGGS
OF TRITURUS TOROSUS

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G. MERLIN GOOD and J. FRANK DANIEL*

INTRODUCTION

FERTILIZATION IN *Triturus torosus* takes place after the egg has passed through the oviduct and has entered the cloaca. At this stage the egg is surrounded by four concentric layers of jelly, which have been added to it in its passage down the oviduct (Daniel, 1937). These are an inner semisolid layer contiguous to the egg, a second somewhat more solid envelope covering the first layer, a third thin compact layer surrounding the second, and, finally, a viscous outer layer which, after the egg enters the water, increases enormously in thickness.

The egg itself at the time of fertilization has undergone marked internal changes. In the peripheral cytoplasm of the animal pigmented cap the yolk plates are small and variable in number, but they are not infrequently horizontal in position and orderly in arrangement (see Daniel and Yarwood, 1939, fig. A). In the periphery of the vegetative area the plates have become medium or small in size. In fact, only in the lower part of the central mass of yolk are the plates still large. The second maturation spindle, which was formed early, is still intact, with one end abutting against the ani al pole of the egg. In the event a sperm cell has gained entrance into the cloacal egg, the second polar body may now be in the process of formation.

THE COELOMIC EGG AND ITS FERTILIZATION
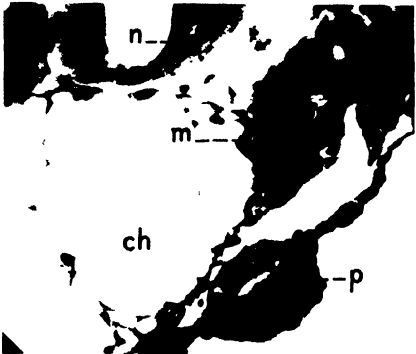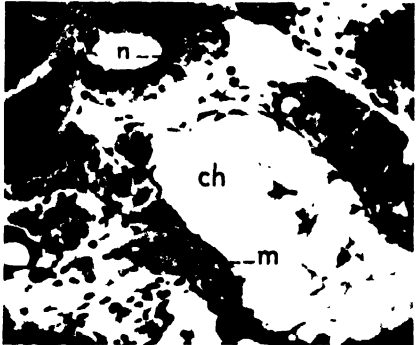
The coelomic egg, on the other hand, presents a pattern differing in several respects from that described for the egg which has reached the cloaca and is ready for fertilization. As the egg enters the body cavity, or coelom, it possesses none of the jelly layers present around the cloacal egg. In the peripheral cytoplasm of the vegetative area it may still possess remnants of the thin zona radiata characteristic of the ovarian egg, and in this area also the peripheral yolk plates are of large size as compared with those of the cloacal egg (Daniel and Yarwood, *loc. cit.*). In the central region large plates constitute the so-called white yolk.

At this stage the early activities of maturation take place, initiating changes in the nuclear material and cytoplasm and producing formed structures some of which we have observed in the cloacal egg. At the time the ovum enters the body cavity the large germinal vesicle is still intact, but its walls break down and the nuclear sap mixes with the cytoplasm. A first maturation spindle then forms, on which the chromosomes appear; a first polar body is extruded from the egg; and the second maturation spindle is produced either in the coelom or in the upper part of the oviduct. This second spindle, as we have seen, persists as the egg passes down through the oviduct to the cloaca, where, after sperm penetration, the second polar body is given off.

Before studying the fertilization of the coelomic egg, which is devoid of jelly coats, eggs in the other segments of the oviduct were examined. All eggs even in the lowest part of the oviduct, when removed for examination, proved to be unfertile. The

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* The latter since deceased.

addition of a concentrated suspension of sperm cells, however, to eggs from this segment, or to eggs from any other segment which had any of the jelly coats around them, resulted in activation and segmentation.

EXPERIMENTATION

From 23 *laying* females 32 coelomic eggs were removed and placed in a modified Ringer's solution which was made as nearly isotonic with the amphibian coelomic fluid as possible. The eggs were then transferred to a small amount of spring water to which a dense sperm suspension was added. In this they remained for 30 minutes. One of these eggs (3 per cent, see table 1) showed signs of segmentation. From the same 23 females 78 more coelomic eggs were taken. To these eggs jelly from the upper segment of the oviduct was added, and the eggs were similarly placed in a sperm suspension. Thirty of these eggs (38.4 per cent) were fertilized and underwent segmentation.

TABLE 1

COELOMIC EGGS IN SPERM SUSPENSION

No. of ♀♀	Source of eggs	No. of eggs	Treatment of eggs	No. of eggs cleaved	Per cent cleaved
23	Laying females..........	32	No treatment	1	3.0
		78	Jelly-covered	30	38.4
13	Pituitary-injected fe-males................	129	No treatment	3	2.3
		289	Jelly-covered	81	28.0

The next experiments were made on females which were induced to lay by injections of a water extract of *Triturus* pituitary, either added subcutaneously or put directly into the body cavity. Twelve to 15 hours after the first injection of approximately one pituitary per female, a second injection of one-half the value was given. It was observed that eggs which leave the ovary and accumulate in the body cavity are readily seen through the body wall. At the end of 20 to 56 hours the females were opened and the coelomic eggs removed to a modified Ringer's solution, as were the eggs from laying females. The number of coelomic eggs thus obtained per female from pituitary injections ranged from 20 to 75, as contrasted with 0 to 14 from actively laying females. The large number was also found in *Triturus pyrrhogaster* by Streett (Streett, 1940). In *Triturus torosus* the increase in number may be explained by the fact that of the many eggs which left the ovary some were of smaller size, suggesting that they had left the ovary at an earlier stage than do the eggs in normal ovulation.

From 13 females injected with pituitary, 129 coelomic eggs were obtained and exposed to a dense sperm suspension. Three of the 129 naked (?) eggs (2.3 per cent, see table 1) when thus exposed showed signs of cleavage. Two hundred eighty-nine additional coelomic eggs from the same 13 females were first covered with jelly, and when these were similarly exposed to a sperm suspension 81 (28 per cent) underwent early development.

In these studies, a few naked (?) coelomic eggs, both from normally laying females and from females which were induced to lay by pituitary injections, showed signs of activation when exposed to sperm suspensions. When coelomic eggs were first surrounded by oviducal jelly and then exposed to sperm suspensions, a rela-

tively high percentage of the eggs, 38.4 per cent in laying females and 28 per cent in injected females, underwent cleavage, showing that they had been fertilized.

Jelly from any of the segments of the oviduct, or a mixture of all the different jellies, when added to the coelomic eggs, appears to aid the sperm cells in their penetration of the eggs; and it is a question whether the dense sperm suspension may not have aided in some similar way the few sperms which entered the naked eggs.

FURTHER DEVELOPMENT OF COELOMIC EGGS

At the time of sperm penetration the conditions within the coelomic egg do not readily permit the activities which normally accompany and succeed fertilization. This might be especially true of younger eggs released from the ovary upon pituitary injection. But it is also true for coelomic eggs released through normal ovulation. As we have seen, these eggs are in the process of completing certain activities essential to fertilization and early development. In some the germinal vesicle has just broken down and the first polar body is in the process of formation; in others the first polar body has just been extruded. In the event the second maturation spindle is not formed until after the egg enters the oviduct, the egg would certainly not be ready for completed fertilization. The sperm may enter the egg, but retardation in forming the second maturation spindle would delay the formation of the egg pronucleus, and consequently, the meeting and interaction of sperm and egg nuclei. These delays would hinder the interactions which constitute fertilization.

In the period following fertilization, delay is also evident. In a group of eggs from the same laying female, after 16 hours one was in the 2-cell stage of cleavage, another was in the 4-cel stage, and two were in the 8-cell stage. Moreover, the average time taken for 23 coelomic eggs from pituitary-injected females to reach the 4-8 cell stage was 26 hours. This is sufficient time for normal eggs to become completed blastulae. In one egg which reached the greatest development of any of the coelomic eggs thus far fertilized, delayed cleavage began on a given day and neural folds appeared exactly one week (instead of 3 days) later. It is evident in this egg that the handicap to the process of fertilization was carried over into and beyond the succeeding period of cleavage.

Irregularities in cleavage pattern may also characterize the developing coelomic egg. In normal cleavage the first three divisions follow a regular plan, resulting in a quartet of blastomeres at the animal pole, and a larger quartet at the vegetative pole. In the segmenting coelomic egg the first two blastomeres, A and B, may or may not be of unequal size. In figure 1, where marked inequality obtains, the first segmentation spindle appears to have taken a horizontal position, but the cleavage furrow for some reason divided the egg unequally. Whether this is to be attributed to the condition of the (superficial) cytoplasm or to obstruction by the yolk is not clear. In figure 4, the cleavage plane divided the egg into nearly equal parts, but after the plane had passed through the animal hemisphere it was deflected from its course and turned sharply upon itself.

Figure 2, which is of the same egg as figure 1, reveals seven irregular blastomeres. Two sperm cells had penetrated the egg, and this might have affected the pattern, but in the cleavages immediately following some of the blastomeres in the animal area extended outward budlike, indicating that the mechanism of division had been interfered with. In the coelomic egg shown in figures 5–6 the two sides of the egg varied greatly in their development.

In later cleavages even in normal development the cleavage spindles and planes are influenced by the heavy internal yolk. After a blastocoel is fully formed, however, the surface blastomeres, both in the normal and in coelomic eggs, become more nearly of a size (fig. 3). Few coelomic eggs inseminated attained the degree of development shown in this figure, and only one, as we have seen, reached the neural stage.

SUMMARY

1. Eggs removed from the cloaca of *Triturus torosus* are usually found to be fertile or are readily inseminated. Coelomic eggs, if covered with oviducal jelly and exposed for 30 minutes to a sperm suspension, may also be fertilized.

2. The coelomic egg at this time may have completed its first maturation with the extrusion of the first polar body; and the second maturation spindle may form now or it may not be formed until the egg enters the oviduct. The incompletion of these processes, together with a lack of preparation both in the cytoplasm and its contained yolk, results in retardation which is revealed by a delay in the time at which cleavage begins and the rate at which it proceeds.

3. The greatest development attained by fertilized coelomic eggs was the neurula stage; and this stage required one week at room temperature for its appearance.

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EXPLANATION OF PLATE

Figs. 1–3. Three stages in the segmentation of the same coelomic egg of *Triturus torosus* (top view). 1, Two-cell stage with blastomeres unequal in size. 2, Eight irregular blastomeres. 3, Blastula.

Figs. 4–6. Segmentation of two coelomic eggs of *Triturus torosus* (side view). 4, Two blastomeres of equal size but cleavage incomplete. 5–6, Different sides of the same egg to show differences in rate of cleavage and cleavage pattern.

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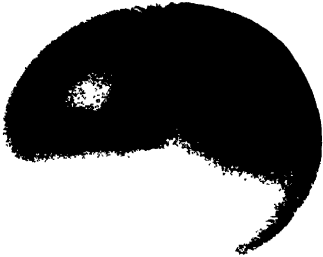
REGULATION IN THE
ENTODERM OF THE TREE FROG
HYLA REGILLA

BY

NORMAN E. KEMP

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CONTENTS

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TREE FROG, *HYLA REGILLA*

BY

NORMAN E. KEMP

INTRODUCTION

RELATIVELY little has been published on the developmental mechanics of amphibian entoderm, in contrast to the volume of literature dealing with the development of ectodermal and mesodermal structures; and many problems concerning organization and regulation within the entoderm remain unsolved. When do the presumptive epithelial cells of the esophagus, stomach, or intestine become irreversibly determined? Is there a period of labile determination during which environmental influences may bring about regulation? If entodermal regulation occurs, how and to what degree is it evident?

It is well to realize at the outset that regulation is a general term and that there are many kinds of regulation. In its broadest contemporary usage, it refers to any change in the path of differentiation of an embryo or embryonic rudiment in response to an altered environment. Such a change may result in an adjustment leading to normal differentiation of the part in question (Driesch), or it may reveal developmental potentialities never realized in normal development. In any case, the nature and degree of a regulatory change are dependent upon the stage of differentiation of the part and upon the spatial relations which prevail at the time an altered environment becomes operative (see Gilchrist, 1933). Prior to gastrulation, before the germ layers become established and their separate anlagen determined, regulation may produce changes of greater magnitude than at stages subsequent to gastrulation. Presumptive ectoderm from the early gastrula, for example, may be transformed into mesoderm (Mangold, 1923). After the various fields of the embryo have been established, however, regulatory phenomena are limited to modifications in the development of form within these histologically determined areas. Within the limb field, for example, regulation may bring about changes in the development of the limb (Harrison, 1918), but it cannot transform presumptive limb cells into cells of any other type.

Holtfreter (1938a) distinguishes three different types of regulation: (1) "morphological regulation," simple rearrangements and increase or decrease of material of a particular kind; (2) "histological or material regulation," a change in the histological fate of a given rudiment, as, for example, a change of presumptive chorda to neural tube; (3) "assimilative regulation," certain formative influences such as fusion with, or invasion by, bodily parts not normally concerned with the development of the structure in question. An example of the last type would be the harmonious fusion of buccal and intestinal epithelia to form a simple, continuous digestive tube, as described by Born (1897). Histological regulation, or "qualitative regulation" (Eakin, 1939), may take place during the early stages of development before histological determination of a given rudiment has become irreversible. Morphological or "quantitative" regulation (Eakin, *loc. cit.*) may be effective until much later, but can bring about changes only in the form of the developing organs. The present study deals with the ability of the entoderm of the Pacific tree frog, *Hyla regilla*, to regulate both histologically and morphologically.

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I wish to express my gratitude to Professors J. Frank Daniel and Richard M. Eakin, under whose direction the research was conducted. I wish also to acknowledge the helpful suggestions of Dr. A. B. Burch and Mr. J. E. Gullberg in connection with certain problems of photography and microtechnique.

MATERIALS AND METHODS

The Pacific tree frog, *Hyla regilla,* is an especially favorable amphibian for studies in experimental embryology. In the first place, its eggs are available for a relatively long period of time, from early January until the first part of July. Second, the entoderm of *Hyla* offers the advantage of solid consistency, which facilitates accurate microsurgery. This feature was indispensable for the success of the experiments reported here. Furthermore, the egg is relatively small, from 1 to 2 mm. in diameter at the end of gastrulation, and this facilitates estimating the position of the planes of transection.

All experiments here described were performed on the early or middle neurula. Embryos were cut with iridectomy scissors into parts which included definite sections of entoderm. These parts were variously joined so that the amount of entoderm in the resulting embryo was in some instances smaller; in others greater, than in the norm; and, in still others, the entoderm was abnormally oriented.

Parts of embryos to be fused were placed together in a mold of wax in the bottom of an operating dish and the cut surfaces held together by pressure applied to the sides of the pieces by means of thin sections of coverglass flamed at one end to give a rounded surface. Adjustments in pressure were made by varying the dimensions of the mold. Two or three hours were usually allowed for healing, although an hour was often sufficient. If healing was incomplete after three hours, it was often necessary to scarify the cut edges of the pieces being fused and to readjust the pressure.

The operations were performed in sterilized Holtfreter's solution diluted with an equal amount of distilled water. The embryos were cultured separately in the same medium for two or three days; thereafter Holtfreter's solution was replaced by pond water. The period of culture varied from one to three weeks, sufficient time to permit the differentiation of the digestive organs. Animals from which the yolk had been resorbed were fed on strained spinach. The presence of fecal material in the culture dish indicated that the digestive tract was functioning.

Photographs of living embryos and of dissections of the viscera were taken with a Leica camera mounted above an adjustable metal tube. Specimens were fixed either in Bouin's fluid or in a mixture of two-thirds Bouin's fluid to one-third dioxan and stored either in 70 per cent alcohol or in dioxan for periods of three to six months. Sections were cut at 8μ and stained with Harris's haematoxylin and eosin. The photomicrographs were taken with an ordinary photomicrographic apparatus, including an apochromat 16 mm. objective.

NORMAL MORPHOLOGY OF THE LARVAL DIGESTIVE TRACT
MACROSCOPIC ANATOMY

The normal asymmetry of the digestive organs may be observed in an 18-day larva in which the ventral body wall has been partly dissected away to expose the viscera (pl. 16, fig. 10). The stomach (*s.*) and duodenum (*d.*) may be seen on the right side of the body, and the coiled small intestine on the left side. The outer coil of the intestine (*i.o.*), consisting usually of two and a half turns, runs in a counterclockwise direction; the inner coil runs clockwise. The former is connected to the duodenum, the latter to the large intestine, which lies against the dorsal body wall and runs caudad as a straight tube which opens into the cloaca. The pancreas (*p.*) can

be seen between the stomach and the duodenum. The liver (*l.*), a part of which can be seen in the angle between the stomach and the small intestine, borders on the esophagus dorsally and the transverse septum anteriorly.

<div align="center">MICROSCOPIC ANATOMY</div>

The histological features of the digestive tract of *Hyla regilla* are well established in the 11-day tadpole. The buccal epithelium is composed of a single layer of squamous nonciliated cells; beneath these cells is a loose layer of connective tissue. The epithelium of the pharynx is predominantly cuboidal; at some levels, however, it is squamous. The gill chamber is lined with squamous epithelium, except for a few restricted areas of ciliated columnar cells.

Posterior to the outpocketing of the lung, which marks the caudal end of the pharynx, the ciliated columnar epithelium of the esophagus begins. Anteriorly, the esophagus consists of a smooth lining of epithelial cells enveloped closely by a thin layer of peritoneum; posteriorly, however, the epithelium becomes folded and a definite lamina propria separates the epithelium from the peritoneum.

The stomach has the most complicated structure of any of the regions of the digestive tract. The epithelium is characterized by high, ciliated columnar cells and many folds. Beneath the epithelium is a relatively thick lamina propria occupied almost entirely by simple tubular glands, branched or unbranched.

The outer intestinal coil has a distinctive cylindrical epithelium with a conspicuous striated border; the inner coil has a much thinner, cuboidal epithelium. The posterior part of the large intestine and the anterior part of the cloaca possess a unique type of ciliated epithelium. Here the cells are roughly cuboidal, with a convex surface next to the lumen. Furthermore, the cells, and especially their nuclei, are slightly elongated in a direction parallel to the length of the lumen. Squamous cells line the posterior part of the cloaca.

There are several criteria for identifying liver and pancreas. The first is relative position: the liver extends farther anteriorly, the pancreas farther posteriorly; this criterion is useful when a complete series of sections is available. Second, the cells of the liver are arranged in cords surrounded by many blood cells. In contradistinction, those of the pancreas are arranged in acini, and there is much less blood than in the liver. The two organs differ, moreover, in the size, shape, and staining reaction of the nuclei. In the liver the nuclei are uniform in size and shape, have usually a single concentrated karyosome, and tend to stain more lightly with haematoxylin than do those in the pancreas. This staining property of the liver is especially marked in poorly fixed material. The nuclei in the pancreas are of different sizes and shapes, often possess a diffuse karyosome, and stain unevenly with haematoxylin. The gall bladder has a simple cuboidal epithelium.

<div align="center">EXPERIMENTAL PROCEDURE AND RESULTS</div>

<div align="center">EXPERIMENT 1. FUSION OF LATERAL HALVES OF EMBRYOS IN REVERSED ANTEROPOSTERIOR DIRECTION (*Re* SERIES)</div>

Procedure.—Two early neurulae were cut sagittally. The two right halves were then fused together in a reversed anteroposterior orientation (fig. A), the two left halves likewise. Anterior parts of the digestive tract thus came to lie in association with posterior parts. Presumptive cloacal cells, for example, were placed in contact with presumptive buccal cells; materials normally forming pharynx, esophagus, and stomach adjoined presumptive intestine.

This experiment was designed to test the influence of one region of undifferen-

tiated entoderm upon another. Can one entodermal anlage induce or inhibit the differentiation of a second so that histological regulation may take place?

Results.—250 operations; 51 animals (approximately 20 per cent) fixed 3–20 days later.

1) Gross development. Fusion was rendered difficult at the two ends of the reversed halves by the greater length of the foregut as compared with that of the hindgut, although the embryo was nearly spherical at the time of the operation. During the first few hours healing was often incomplete wherever hindgut and foregut were in contact. Later, however, these regions frequently did come together and fuse. In the region of the midgut, fusion usually took place soon after the operation, if the pressure of the bridges and mold was carefully adjusted. The medullary plate in the two halves healed together quickly, as did also the chordamesoderm.

On the third day of culture the tail at each end had become pointed and often curled at the tip. In some specimens one tail developed faster than the other and became more active in swimming movements. The heart at each end of the coelom was beating by the third day in those specimens which had healed well in the region of contact between foregut and hindgut.

During subsequent days the two sides of the animals continued to differentiate autonomously in external features. One particularly instructive specimen, fixed 12 days after the operation, has such conspicuous dorsal features as the single eye (*e.*, pl. 15, fig. 1) and tail (*t.*) on opposite sides at both ends of the embryo; also, an unpigmented area (*br.*) at the base of each tail, directly dorsal to the forebrain. An external naris (*n.*) can be seen on one side just anterior to the eye. In the living animal an ear could be seen behind each eye. Ventrally, a mouth (*m.*, pl. 15, fig. 2) is present at each end, bordered externally by horny teeth and beak; these structures represent only half of the normal armament and occur only on the side bearing structures of the head. The pigmented oval area (*c.*) extending transversely across the ventral surface delimits the coelom. The white gut (*g.*) shows through the body wall. A well-developed, pulsating heart just anterior to the transverse septum was present in each half of this specimen, and circulation was good. There are separate openings for mouth and anus at one end of the embryo, a single opening for both at the other end. The gut itself is a relatively straight, uncoiled tube.

Fig. A. Diagram of operation in Experiment 1 in which lateral halves of neurulae were fused in reversed anteroposterior direction.

The form of the digestive tract in a specimen from which the ventral body wall has been removed is shown in plate 15, figure 3. Liver (*l.*), pancreas (*p.*) and gall bladder (*g.b.*) may be seen at one end of the tract; liver appears also at the opposite end. Except for a lobe of intestine (*i.*), the digestive tract proper is a smooth tube; there is no line of demarcation to show the limits of the two halves which contributed to it.

2) Histology. In 19 of the 51 embryos the various organs of the digestive tract were well differentiated histologically. One animal, fixed 11 days after operation, has been selected for description and illustration. Structures anterior to the stomach are well differentiated, as are also the posterior intestinal and cloacal epithelia. The stomach and major part of the intestine, however, still possess many yolk platelets. Glands are present in the wall of the stomach, but the epithelial cells which line the

lumen have not yet developed cilia. The wall of the intestine is much thicker than in a control larva of the same age, and the epithelium exhibits pseudostratification. The pancreas is also incompletely differentiated; yolk platelets are abundant in all the cells. Plate 15, figures 4–9 illustrate these and further details.

A section from one end of the embryo illustrates the region in which buccal epithelium (*b.*, fig. 4) and intestinal epithelium (*i.*) have developed in contact. Note the sharp line of demarcation (*x.*) between the two types of epithelium. A Wolffian duct (*w.d.*) may be seen to the right of the boundary between oral and intestinal walls. The folding of the intestine into the lumen is the only evidence that the amount of intestinal material available for the formation of gut at this level was greater than the amount of presumptive oral epithelium.

Proceeding toward the middle of the embryo from the level represented by the preceding figure, the lining of the digestive tract presents an entirely different picture (see fig. 5). Here the epithelium on the left dorsolateral side of the tract is pharyngeal (*ph.*) and esophageal (*o.*), that on the right side, intestinal (*i.*). There is a section of stomach (*s.*) between the esophagus and the intestine. The liver (*l.*) is present beside the intestine; still farther to the left lies the heart (*h.*). In the upper part of the photomicrograph may be seen two notochords and a section of spinal cord adjacent to brain.

A section near the middle of the animal is shown in figure 6. Here the entire digestive tract is composed of intestinal epithelium. Two dorsal mesenteries (*m.*), which may be seen converging to form a single mesentery, are present above the intestine. A large lobe of the liver (*l.*) also lies dorsal to the intestine (*i.*). Between the liver and the intestine is the bile duct (*b.d.*). Observe that both the notochord and neural tube are single in this section, indicating the complete fusion of the two halves of these structures brought together by the operative technique.

At a level of the embryo corresponding to that illustrated in figure 5, but at the opposite end of the body, may be noted the following types of epithelium: intestinal (*i.*, fig. 7), gastric (*s.*), esophageal (*o.*), and pharyngeal (*ph.*). Here, however, the arrangement of the types is the bilateral reverse of that shown in figure 5. Sections of the heart (*h.*) and of one of the aortic arches appear to the right of the gut.

A section nearer the end of the embryo than that represented in figure 7 shows intestinal epithelium (*i.*, fig. 8) on the left side, and buccal epithelium on the right. Note that this picture is the bilateral reverse of that shown in figure 4. The final section chosen for this series (see fig. 9) shows only buccal epithelium lining the oral cavity. The larval beak (*bk.*) and stratified squamous epithelium (*sq.*) of the lip appear on the right external margin of the mouth.

<div style="text-align:center">EXPERIMENT 2. EXTIRPATION OF ENTODERM</div>

Procedure.—Wedge-shaped blocks consisting of entodermal, mesodermal, and ectodermal cells were excised from the ventral region of the early neurula, in three types of operation (fig. B): (1) section *Li* includes approximately the anterior fourth of the mesenteron; (2) a larger block, section *Lim*, consists of the anterior third of the midgut; (3) section *Int* includes the middle third of the mesenteron. In each operation the extirpated material was discarded and the cut surfaces of the remaining anterior and posterior parts of the embryo allowed to heal together.

These experiments were performed to test the degree and type of regulatory compensation for lost materials. Is a replacement of these materials made from entoderm of different presumptive value (histological regulation), or is there merely a rearrangement or regeneration of entoderm of like presumptive value (morphological regulation)?

Results.—The results of extirpation of entoderm from the three regions of the mesenteron illustrated in figure B are markedly different.

1) Removal of anterior fourth of mesenteron (*Li* series). Forty-two operations; 22 embryos fixed. Eleven of the 22 regulated completely; the remaining 11 had abnormally coiled intestines, although all but 1 had a well-developed liver and pancreas. In 3 specimens the liver projected through the transverse septum into the pericardial cavity. Seventeen specimens, or 77 per cent, had functional tracts.

2) Removal of anterior third of mesenteron (*Lim* series). Forty-three operations; 23 specimens fixed. In each of these the digestive tract was so abnormal as to be non-functional. There was usually a great reduction in the coiling of the intestine. Nineteen embryos exhibited an S-shaped digestive tract (*Lim* 14 and *Lim* 15, pl. 16, figs. 11 and 12). In addition to the reduction in intestinal coiling, there was also in most of these specimens a shortening and broadening of both esophagus and stomach. Sections of *Lim* 15, for example, reveal that the pharynx opens directly into the stomach. Although there is no region which may be designated as the esophagus, it is noteworthy that esophageal epithelium does occur between pharynx and stomach on both lateral walls of the tract. The liver and pancreas, even though much reduced in size, could be positively identified in all but 2 specimens. Other features common to most of these embryos were absence of circulation; tubular, uncoiled heart; transverse septum absent or incomplete. Four specimens had digestive tracts coiled more than the S-shaped tract exemplified in *Lim* 14 or *Lim* 15.

Fig. B. Diagram showing the types of operation in Experiment 2. *Li,* anterior fourth of mesenteron extirpated in one series; *Lim,* anterior third of mesenteron removed in a second series; *Int,* middle third of the midgut excised in a third series.

It is significant that in these 4 the transverse septa were well developed, the hearts pulsated normally, and circulation was good. The importance of the transverse septum for normal development of the heart and digestive tract will be discussed in a later section.

3) Removal of middle third of mesenteron (*Int* series). Fifty-four operations; 37 embryos fixed 8–17 days later. Thirteen, or approximately one-third, regulated completely; 34, or 92 per cent, had functional tracts. Included among those showing complete regulation were 3 specimens in which, although the intestinal coiling was

TABLE 1

RESULTS OF EXTIRPATION

Series	Number in series	Per cent showing complete regulation	Per cent with functional tracts	Abnormality of liver and pancreas
Li.....................	22	50	77	Relatively slight; liver perforates transverse septum in 3 larvae
Lim....................	23	0	0	Both organs much reduced in size in all but 4 larvae
Int....................	37	35	92	None

normal in position, the number of turns in the coil was slightly reduced. Coiling of the intestine was abnormal in the remaining 24 animals; stomach, liver and pancreas, however, developed normally in all of these. (See table 1.)

EXPERIMENT 3. ADDITION OF ENTODERM BY FUSION OF ANTERIOR AND POSTERIOR PARTS OF EMBRYOS

Procedure.—Transections of two mid-neurulae were made at approximately the following levels (see fig. C): (1) the boundary between foregut and mesenteron; (2) the posterior limit of the first third of the midgut; (3) the middle of the mesenteron; and (4) the anterior limit of the posterior third of the midgut. The anterior part of one embryo was then fused to the posterior part of the other so that a given section was duplicated.

Four different regions of entoderm were duplicated in these experiments. In one series (*Mo*) the anterior piece of an embryo cut on line 2 (see fig. C) was joined to the posterior piece of an embryo cut on line 1. Thus the region between levels 1 and 2 was duplicated, as shown by the cross-hatching in the figure. This anterior third of the midgut presumably contains the anlagen of the liver and pancreas. Similarly, the region between levels 1 and 3, namely, the first half of the midgut, was duplicated in a second series (*Mo'*). In the third series (*Mo''*) the first two-thirds of the midgut was duplicated. Finally, the region between levels 3 and 4 was reproduced in a fourth series (*Mi*). Posterior parts of embryos cut along line 1 were cultured in a control series.

Fig. C. Diagram showing the types of operation in Experiment 3. Levels at which neurulae were transected shown by lines 1, 2, 3, and 4. *Mo*, anterior third of mesenteron duplicated in one series; *Mo'*, anterior half of midgut duplicated in a second series; *Mo''*, first two-thirds of midgut duplicated in a third series; *Mi*, posterior segment of midgut duplicated in a fourth series.

These experiments were designed to show the degree and kind of regulation when various levels of the midgut were abnormally associated with another level.

Results.—1) Duplication of anterior third of mesenteron (*Mo* series). Thirty-nine operations (see fig. C); 25 embryos examined subsequent to differentiation of the digestive organs. Twelve (48 per cent) larvae regulated completely except for slight variations from the normal in the size of the liver, gall bladder, or pancreas. Plate 16, figure 13, shows a typical larva in which the digestive organs are practically normal. The middle of the liver, however, is larger than in the norm; note also that there are only one and a half turns in the outer intestinal coil. Coiling of the intestine was very irregular in the 13 specimens classified as abnormal. Twenty (80 per cent) animals had functional tracts. Although each embryo possessed two hepatic and two pancreatic anlagen as a result of the operation, only the pancreas was duplicated at the time of fixation. In 1 larva the pancreas was completely double; in 4 others it either possessed two lobes or was considerably enlarged. The tendency, observed in several specimens, toward duplication of the gall bladder provides the only evidence that two anlagen may have shared in the formation of the liver. Four
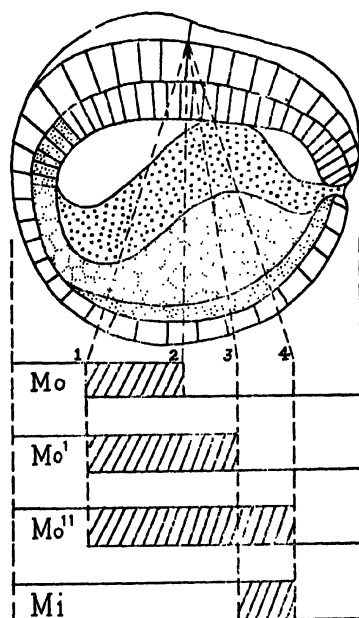
and pancreas were all well formed in these 4 specimens, but were extremely abnormal in the remaining 4. Functional tracts occurred in 3 of the 9 animals.

The photographs of 2 of the most abnormal specimens (pl. 16, figs. 20 and 21) show the extreme irregularity in the anterior viscera. The stomach (*s.*, fig. 20) is far to the left in one specimen, and the liver (*l.*), on the right, is widely separated from the pancreas (*p.*). In the other animal (fig. 21), the stomach is an enlarged sac to the right, connected to the intestine on the left anterior side. Liver and pancreas occur, as is normal, on the right side of the body, but even in this specimen the liver is anterior to the pancreas. Sections of these 2 abnormal specimens revealed a great reduction of the esophagus. Correlated with this is the incomplete development of the transverse septum, as was true in most of the animals of the *Lim* series previously described. The pharynx and stomach are almost directly continuous, since esophageal epithelium is restricted to a narrow band in the lateral walls of the gut.

Fig. D. Diagram showing the types of operation in Experiment 4. *Liv,* the anterior third of midgut rotated in one series; *Int,* middle third of midgut rotated in a second series.

Of the 4 control animals in this series, only 1 was completely normal. The other 3 showed abnormalities in the form of the duodenum, but the other organs of the gastroduodenal region were relatively normal. Moreover, the relative position of all digestive organs was normal in all these animals. It is believed, therefore, that excision alone, without rotation, cannot be considered sufficient cause for abnormalities of the degree noted in the 4 most abnormal specimens of the group undergoing rotation.

2) Rotation of middle third of mesenteron (*IntRev* series). Thirty operations; 18 animals dissected in 8–15 days. The major difference between this and the preceding series is merely one of the position along the digestive tract at which abnormalities occur. In the preceding series the anterior viscera showed the greatest irregularities; in the series now to be described the abnormalities were for the most part in the intestine (see pl. 16, fig. 22). Stomach, liver, and pancreas usually developed normally, except for minor shifts in position. Despite the rotation of a third of the presumptive intestine, the intestinal coil occurred on the right side in 1 larva only. Coiling was considerably reduced in 5 specimens (pl. 16, fig. 23). Two animals regulated almost completely (pl. 16, fig. 24); 4 others were nearly normal. Eleven animals (61 per cent) had functional digestive tracts, about double the number in the *LivRev* series. This difference assumes importance

TABLE 3

RESULTS OF ROTATION

Series	Number in series	Per cent showing complete regulation	Per cent with functional tracts	Abnormality of liver and pancreas
LivRev..................	9	11 .	33	Form of both abnormal in 44 per cent; relative position normal in 100 per cent
IntRev..................	18	11	61	None

when compared with the other results described in this study. Reference to tables 1 and 2 will reveal that whenever stomach, liver, and pancreas tended to be abnormal (*Lim, Mo'* and *Mo"* series) the percentage of functional digestive tracts was much lower than when the abnormalities were largely confined to the intestine (*Li, Int, Mo,* and *Mi* series).

The results of Experiment 4 are summarized in table 3.

CONCLUSIONS AND DISCUSSION
HISTOLOGICAL REGULATION OF AMPHIBIAN ENTODERM

The results reported herein support Holtfreter's general conclusion that the entoderm of amphibians cannot regulate histologically. Since the experiments here described were performed at the neurula stage, they do not specifically substantiate Holtfreter's belief that entoderm is regionally determined even in the early gastrula; neither do they directly disprove the thesis supported by Kusche (1929) and Balinsky (1938) that the entoderm of the early gastrula is determined only in a labile fashion, if at all. Two of these experiments do, however, offer strong resistance to Balinsky's claim that the entoderm may regulate histologically in the neurula.

In the first of the experiments (*Re* series), lateral halves of neurulae were joined so that the anterior end of one half was laterally opposite the posterior end of the other half; thus anterior and posterior entodermal derivatives, such as buccal and intestinal epithelium, developed in contact. Each epithelial type differentiated, nevertheless, in accordance with its origin, not in accordance with its environment. Although a single tube resulted from the fusion of the two halves, one side of the tract was composed of intestinal epithelium, the other side of buccal epithelium. There was no evidence that entoderm from one level induced or inhibited the differentiation of entoderm from any other level of the neurula.

Similarly, another experiment demonstrates the self-differentiating character of the entoderm of the neurula. In this experiment (*Mo, Mo'* and *Mo"* series), anterior and posterior parts of embryos were fused in such a way that two anlagen of the stomach, liver, and pancreas were separated by varying amounts of presumptive intestine. As an example of the behavior of entoderm in foreign environments, it is of interest to consider the differentiation of the posterior gastric anlage. In the *Mo* series, in which the duplicated anlagen were close together, a secondary stomach did not develop in any of the embryos. A secondary stomach did, however, develop in several animals of the *Mo'* series, and in nearly all of the *Mo"* series. These results indicate that when two anlagen of the stomach are contiguous, they fuse to form a single rudiment; when prevented from joining, however, as in some animals of the *Mo'* series and most of the *Mo"* series, they differentiate independently. The posterior rudiment differentiates into typical gastric epithelium even though associated with a section of intestine posterior to the duodenum. There was no evidence of histological regulation on the part of either anterior intestine or secondary stomach at their zone of junction.

It is, of course, possible that the apparent contradiction between Balinsky's results and mine are a consequence of the different methods and animals used. Although entoderm from *Triton* or *Amblystoma,* both urodeles, may be able to regulate histologically in the neurula under the conditions of Balinsky's experiments, it certainly is not true for the anuran, *Hyla,* under the conditions described in this paper. In like manner, the fact that the entoderm of *Triton* transplanted to the optic cavity can differentiate into chorda, musculature, or mesenchyme, as Kusche (1929) claims, does not necessarily contradict Holtfreter's (1929) state-

ment that entoderm transplanted to the coelom differentiates according to its normal prospective fate (see also Holtfreter, 1931, 1938a, 1938b, and Fischer, 1937). A repetition of the experiments *in vivo* of Holtfreter, Kusche, and Balinsky, by the same methods and with the same or similar material, is essential before definite generalizations can be made about the limits of histological regulation of the entoderm in Amphibia.

It is known from the work of Hunt (1937) and Rudnick and Rawles (1937) that the entoderm of the chick is determined relatively late, namely, at the beginning of the stage of the head process. If Holtfreter is correct in his conclusion that amphibian entoderm is irreversibly determined in the early gastrula, a major difference exists between amphibians and birds in the morphogenesis of entoderm. Mangold (1923) attributes the apparent inability of entodermal cells of amphibians to transform into mesodermal or ectodermal structures to the large amount of yolk present. The idea that histological regulation of the entoderm is mechanically impossible is quite different from Holtfreter's view that the failure of entoderm to regulate histologically depends upon a fundamental cytoplasmic determination some time before gastrulation.

MORPHOLOGICAL REGULATION OF AMPHIBIAN ENTODERM

The great ability of amphibian entoderm to regulate morphologically, in contrast to its limited power of histological regulation, has been demonstrated in this study in each of the four types of experiment performed.

1) Formation of a continuous digestive tube from entodermal anlagen experimentally brought together. This type of morphological regulation, first shown by Born in 1897, has been further demonstrated in the following experiments. First of all, it was pointed out that a single, continous tube developed in the embryos resulting from fusion of two lateral halves in reverse orientation (*Re* series). Instead of differentiating into the form which it attains in normal development, the entoderm in each half became adjusted to fit the size and shape of that of the other half. The half of an intestine on one side, joined to the half of a mouth, pharynx, and esophagus on the opposite side, remained uncoiled and straight except for the slight folding noticed in some specimens. Second, a single digestive tube developed in those embryos in which the anlagen of the stomach, liver, and pancreas were duplicated (*Mo, Mo'* and *Mo''* series). Gastric epithelium from the posterior anlage united harmoniously with the intestine from the anterior part of an experimental embryo, yet each epithelial type retained its distinctive histological characteristics.

The smooth union of the two types of epithelium at their zone of junction may be classified in Holtfreter's terminology as assimilative regulation. In this sense both types are "assimilated" into a common structure, the digestive tube. We are, however, considering as this first type of morphological regulation the adjustments in form which the contributing anlagen must undergo to produce a unified structure.

2) Attainment of the normal form of the tract after extirpation of entoderm (*Li, Lim,* and *Int* series). Despite the removal of part of the entoderm in animals of the *Li* and *Int* series, the digestive tract frequently developed normally. This is actual regulation as Driesch (1891) conceived it—retention of the normal pattern of organic form in spite of the resistance offered by environmental forces. Complete regulation took place in 50 per cent of the embryos from which presumptive liver and pancreas had been partly removed (*Li* series) and in 35 per cent of those from which presumptive intestinal entoderm had been extirpated (*Int* series). Holtfreter (1939) has shown that regulation of form can follow ablation of entoderm from the midgut of the tailbud embryo.

Two possible explanations for the ability of the animal to compensate for losses of entodermal material suggest themselves: (1) An increase in the rate of mitosis enables residual entoderm to compensate for the deficiency in the number of cells; (2) the residual entoderm is rearranged over a relatively greater area inside the mesodermal sheath. Now, if the latter were true, one would expect to find the epithelium thinner in the experimental animal than in the normal larva, since fewer cells would be available to form the mucosa. But no striking differences were found. The first explanation, therefore, seems the more probable, especially in view of Holtfreter's statement (1939) that the normal mechanism for the elongation of the epithelium of the gut is a rapid rate of mitosis in the entodermal cells. Such an acceleration in the rate of cell division might well result from the fact that there are fewer than the normal number of cells when active elongation begins, an abnormal strain being thereby imposed upon the residual cells in their adjustment to the developing mesodermal structures associated with the gut.

3) Development of a single organ after fusion of two separate anlagen of like presumptive meaning. Unequal anterior and posterior parts of embryos were fused so that certain entodermal anlagen were duplicated. Comparative figures, presented in table 2, are instructive in interpreting these experiments. In the first place, complete regulation occurred in 48 per cent of the embryos in the *Mo* series, in 5.5 per cent of the *Mo'* series, and in 0 per cent of the *Mo''* series. Second, the pancreas was duplicated or enlarged in 20 per cent of the embryos of the *Mo* series, 62 per cent of the *Mo'* series, and 100 per cent of the *Mo''* series.

It is apparent that the distance between the duplicated anlagen in these series had a decided influence on regulation. The greater the distance separating them, the more difficult for duplicated rudiments to combine and share in the development of a single organ. When the anlagen of the pancreas, for example, were close together, as in the *Mo* series, they could usually combine to form a single pancreas. Fusion of the two anlagen became more difficult in the *Mo'* series and impossible in the *Mo''* series. Similarly, the differentiation of stomach (see above, p. 169) was correlated with the distance between the gastric anlagen.

In this third type of morphological regulation, therefore, a single organ develops from two separate primordia. Such a differentiation is analogous to the development of a single embryo of *Triton* from two fused at the time of first cleavage (Mangold, 1920; Mangold and Seidel, 1927). The fact that regulation of form can take place within the anlagen of the stomach and pancreas indicates that a predetermined pattern does not exist within these anlagen, except possibly in a labile condition. Either such a pattern develops at a stage later than the neurula, or environmental influences are the most important factor in the determination of the normal form of the larval stomach and pancreas. Important among these environmental influences are the development of the peritoneal sheath of the digestive tract and of the mesenteries associated with the gastroduodenal region.

4) Retention of the normal polarity of an organ even though its primordium be rotated 180°. A rearrangement of entoderm was effected within a definite section of the mesenteron (*LivRev* and *IntRev* series). In the first series (*LivRev*), despite rotation of the region presumably containing the anlagen of the liver and pancreas, 1 animal out of 9 developed with completely normal digestive organs. Even more significant is the fact that in all 9 larvae the relative position of the liver and pancreas was normal, the liver invariably lying anterior to the pancreas. Following rotation of the central section of entoderm in the second series (*IntRev*), 2 animals out of 18 developed normally. Furthermore, all but 1 of the 18 developed with the intestinal coils on the left, the normal position.

The normal development of the liver and pancreas in the first, and of the intestine in the second, of these experiments indicates that the anlagen of these organs are equipotential. This conclusion substantiates similar claims made earlier by Holtfreter, Noka, and Yamada. Proof that the anlage of the liver is not regionally determined in the tailbud stage was first presented by Holtfreter (1925). Later, both Noka (1930a) and Yamada (1933b) agreed that in *Bufo vulgaris japonicus* the presumptive liver is equipotential at the tailbud stage. It is not surprising, therefore, that presumptive liver seems to be equipotential in *Hyla regilla* at the neurula stage. Noka (1930b) also published evidence that the anlage of the intestine is equipotential in the tailbud stage of *Rana nigromaculata*. In addition to the experiment discussed in the preceding paragraph (*IntRev* series), two other experiments described in this paper indicate equipotentiality in the intestinal anlage of *Hyla regilla*. The first of these is that in which a section of entoderm from the central part of the midgut was duplicated in an embryo (*Mi* series). One out of 7 animals in this series regulated completely; a continuous intestinal tract developed in all of them. There was no visible line of demarcation in the intestine of any of the larvae to show the region of junction of the two parts joined by the operation. The second additional experiment which shows equipotentiality in the presumptive intestine of *Hyla* is that in which lateral halves were fused (*Re* series). Wherever intestinal epithelium from one half adjoined intestine in the other half, a smooth union took place even though these epithelia might have been derived from different levels of the midgut.

The absence of regional determination within the anlagen of the liver and intestine lends weight to Holtfreter's (1939) view that the form of the gut is plastic and strongly influenced by environmental factors. Holtfreter demonstrated that the entoderm would not form a tube if cultured by itself in neutral salt solution. Upon a mesodermal substrate, however, the entodermal cells did form unmistakable tubes. Holtfreter showed also that even in the late tailbud stage the intestinal epithelial cells have no self-determined polarity; instead, the difference between secreting and basal ends is determined normally by the orientation of these cells with respect to their mesodermal substrate and to the lumen of the gut. That the presence of an organizer may induce the formation of a secondary entodermal tube in connection with the development of a secondary embryo has been shown by Spemann and Mangold (1924) and others. A thorough analysis of the development of form in the primordia of the digestive organs must, therefore, take into account the environmental influences which help to mold the entoderm. These influences include the development of the following structures: the mesodermal sheath of the gut (Holtfreter, 1939), the dorsal mesenteries (Maurer, 1906), the major blood vessels to the gut (Noka, 1930), and the transverse septum.

LOCALIZATION OF THE ANLAGE OF THE LIVER IN THE NEURULA

A general difference which was apparent in the results of the *Mo, Mo'* and *Mo"* series of experiments concerns the amount of duplication of liver as compared to that of pancreas. In contrast to the pancreas, which showed an increasing percentage of duplication with increasing distance between the duplicated anlagen, the liver was single in all animals of the *Mo* series, in all but 2 (6 per cent) of the *Mo'* series, and, likewise, in all but 2 (6 per cent) of the *Mo"* series. These results raise the question, first of all, of the localization of the anlage of the liver in the neurula. Was presumptive liver actually duplicated by the operation in the *Mo, Mo'* and *Mo"* series?

Holtfreter (1925) asserts that the anlage of the liver is situated in the antero-

ventral part of the midgut of the neurula, inseparably adjoining the more posterior anlage of the pancreas. In the tailbud stage the liver is definitely ventral to the anterior end of the midgut, as was demonstrated by Shore in 1891 and Weysse in 1895. At this stage it is already separated from the three anlagen of the pancreas, described by Göppert (1891), Stöhr (1895), and Wolf-Heidegger (1936). The results of extirpating the first part of the midgut of *Hyla regilla* at the neurula stage (*Li* and *Lim* series) leave no doubt that the anlage of the liver is largely within the first third of the floor of the mesenteron. Extirpation of the first fourth of the mesenteron (*Li* series) did not seriously affect the development of the liver; extirpation of the first third (*Lim* series), however, caused a great reduction in the size of both liver and pancreas. It is certain, therefore, that presumptive liver was duplicated in animals of the *Mo, Mo′* and *Mo″* series.

The failure in general of a secondary liver to develop in these animals may be tentatively explained in two ways. First, the two anlagen might have fused to form a single primordium even in the *Mo′* and *Mo″* series, in which the duplicated primordia of the stomach or pancreas usually remained separated. The fact that 4 specimens did have a secondary liver would indicate that in these instances the two anlagen failed to fuse. The occurrence of an elongated or duplicated gall bladder in several specimens of both the *Mo* and *Mo′* series might indicate that the union between the anterior and posterior anlagen was incomplete. In connection with a postulated fusion of the two hepatic primordia, it is important to note that a secondary pancreas or secondary gastric epithelium always developed on the dorsal side of the gut. If the anlage of the liver is situated ventrally and that of the pancreas more dorsally in the anterior part of the midgut, a possible reason for their different behavior in these experiments is at once apparent. It may be that migrations of entoderm on the dorsal side of the gut are mechanically blocked, but that they proceed on the ventral side with relative ease. Under these conditions, union of the hepatic anlagen would result much more frequently than union of the anlagen of the pancreas.

Second, the posterior anlage might have failed to differentiate if separated from the blood supply which the liver normally receives. Holtfreter (1925) showed that the typical histology of the liver is not attained if the anlage of the organ is deprived of blood; liver cells differentiate instead as a solid mass without cords. To determine the fate of the anlage of the liver when isolated from the heart and circulating blood, a series of posterior parts of embryos of *Hyla regilla* were cultured. These parts had been separated from their respective heads by a transverse cut just anterior to the presumptive liver. It is instructive that after differentiation had occurred the liver could be distinguished histologically in but 1 of 6 specimens; more experiments are needed, however, to establish definite conclusions.

THE IMPORTANCE OF THE TRANSVERSE SEPTUM IN THE CONTROL OF ASYMMETRY

Among the extrinsic structures concerned with the normal development of the digestive system, none is more important than the transverse septum. When it is absent in *Hyla regilla,* as in most of the animals of the *Lim* series, the coiling of the intestine is greatly reduced; furthermore, the heart remains straight and tubular, and blood fails to circulate normally. In 4 animals of this series the transverse septum was present, correlated with a more normal development of heart, liver, and digestive tube. Does the transverse septum directly control the development of form in the digestive tract by controlling the development of the mesenteries of the gut, or is its influence indirect through an effect on the development of the liver or heart? Does the absence of the transverse septum directly cause shortening of the gut, or

does it bring about the reduction in length indirectly by upsetting normal circulation? These questions cannot be answered at the present time, but they lead to a theoretical consideration of the role of the transverse septum in the development of the asymmetry of the viscera.

Noka (1930) demonstrated that an injury to the gastroduodenal part of the digestive tract in *Rana nigromaculata* caused abnormal coiling of the entire larval tract. Yamada (1933) showed that extirpation of the liver from one member of a parabiotic pair of toads was followed by shortening and displacement of the gastroduodenal region and a marked reduction in the coiling of the intestine. Noka theorized on the basis of his results that the reversal of asymmetry produced by Spemann (1906), Pressler (1911), and Meyer (1913) after rotation of a dorsal piece of the neurula (see also Wilhelmi, 1921) was due to a reversal in the anlage of the gastroduodenal part of the digestive tube. It is unlikely, however, that reversal of the gastroduodenal region could by itself explain the *situs inversus cordis* which occurs concomitantly with *situs inversus viscerum* in Spemann's experiment. More plausible, in my opinion, is the assumption that the predetermined anlage of the transverse septum is rotated in Spemann's operation.

In reviewing the development of the digestive system, Maurer (1906) described the formation of the transverse septum in *Petromyzon* as follows: a fold forms in each lateral wall of the coelom and grows medially; a secondary fold then develops in the dorsal part of the right primary fold and grows caudally as the dorsal hepatic mesentery which contains the postcaval vein. According to Goette (see Maurer, 1906, p. 225), the development of the transverse septum in Amphibia parallels that in *Petromyzon*. If the dorsal mesentery was made to develop caudally from the left side rather than from the right, the liver, and consequently, the heart, might be expected to develop asymmetrically to the left. With the asymmetry of the liver reversed, it is not difficult to imagine that the stomach might also be pulled toward the left side. Reversed asymmetry in the gastroduodenal region would then, to invoke Noka's view, cause the rest of the tract to develop with reversed asymmetry. Whatever the precise effect of Spemann's operation may be, the transverse septum is probably involved in some manner. The importance of the transverse septum and its associated mesenteries in the determination of symmetry of the heart and digestive organs should be investigated. Demonstration that these mesodermal structures play a decisive part in determining the form of the larval digestive tract in anurans would minimize the importance attributed by Holtfreter (1933, 1939) to an early asymmetrical distribution of the entoderm of presumptive stomach or liver.

SUMMARY

1. The ability of amphibian entoderm to regulate at the neurula stage has been tested in the Pacific tree frog, *Hyla regilla* by: (1) fusion of lateral halves of embryos in reversed anteroposterior direction; (2) extirpation of parts of the mesenteron; (3) addition of entoderm through fusion of anterior and posterior parts of embryos; and (4) rotation of presumptive liver and pancreas or a section of the presumptive intestine through 180°.

2. Confirming Holtfreter's views, it is shown that the entoderm in the neurula lacks the ability to regulate histologically but possesses marked powers of morphological regulation, the terms being used here as defined by Holtfreter.

3. The failure of the entoderm to regulate histologically was revealed in the first experiment. Although anterior entoderm developed in direct association with posterior entoderm, each epithelial type retained its individuality. The self-differentiating nature of the entoderm was further shown by the third experiment.

4. Morphological regulation occurred in each of the experiments, but varied in nature as follows. (1) A single, continuous digestive tube was formed after fusion of the primordia of two histologically different types of epithelium. (2) The normal form of the digestive tract was established after extirpation of the anterior fourth or central third of the mesenteron. (3) In addition to the type of regulation in Experiment 1, a single organ often developed from the fusion of two separate anlagen of like presumptive meaning. (4) The normal polarity of liver and pancreas or of intestine was retained despite rotation of the first third or central third of the floor of the mesenteron. The great ability which the entoderm possesses for morphological regulation in the neurula indicates that each entodermal anlage, although histologically determined, is equipotential within itself.

5. Any consideration of the determination of form in the digestive tract should take into account both the entodermal and mesodermal components of the gut. Furthermore, certain extrinsic structures such as the dorsal mesentery, the blood vessels to the viscera, and the transverse septum are important in establishing the form of the tract. It is suggested that the transverse septum directly or indirectly controls the development of asymmetry in the digestive organs and heart.

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(Magnifications are approximate)

Fig. 1. Dorsal view of *Re 5*, example of Experiment 1, 12 days after operation. *br.*, unpigmented area above brain; *e.*, eye; *n.*, external naris; *t.*, tail. × 10.

Fig. 2. Ventral view of *Re 5*. *c.*, coelom; *g.*, gut; *m.*, mouth. × 10.

Fig. 3. Ventral view of *Re 4*, another example of Experiment 1, dissected to show digestive organs. *g.b.*, gall bladder; *i.*, intestine; *l.*, liver; *p.*, pancreas. × 10.

Figs. 4–9. Photomicrographs of transverse sections cut at successive levels of *Re 8*, a well-differentiated example of Experiment 1. × 25.

Fig. 4. Photomicrograph of a section from one end of *Re 8*. *b.*, buccal epithelium; *w.d.*, Wolffian duct; *i.*, intestinal epithelium; *x.*, line of demarcation between buccal and intestinal epithelia.

Fig. 5. Photomicrograph of a section of *Re 8* at a level nearer the center of the body than that shown in figure 4. *h.*, heart; *i.*, intestinal epithelium; *l.*, liver; *s.*, gastric epithelium; *o.*, esophageal epithelium; *ph.*, pharyngeal epithelium.

Fig. 6. A section of *Re 8* about midway between the two ends. *b.d.*, bile duct; *i.*, intestinal epithelium; *l.*, liver; *m.*, dorsal mesenteries.

Fig. 7. The fourth section in the series selected from *Re 8*. Compare with figure 5. *h.*, heart; *i.*, intestinal epithelium; *o.*, esophageal epithelium; *ph.*, pharyngeal epithelium; *s.*, gastric epithelium.

Fig. 8. The fifth section of *Re 8*, to be compared with figure 4. *b.*, buccal epithelium; *i.*, intestinal epithelium.

Fig. 9. The sixth section in the series from *Re 8*. *bk.*, horny beak; *sq.*, stratified squamous epithelium of the lip.

PLATE 16

(Magnifications are approximate)

Fig. 10. Ventral view of a normal larva dissected to show digestive organs. *l.*, liver; *s.*, stomach; *p.*, pancreas; *d.*, duodenum; *i.i.*, inner coil of small intestine; *i.o.*, outer coil of small intestine. × 7.

Fig. 11. Lateral view of *Lim 14* dissected to show the simple, **S**-shaped digestive tract which developed following extirpation of the first third of the mesenteron. × 8.

Fig. 12. Ventral view of *Lim 15* dissected to show the **S**-shaped digestive tract with its shortened stomach lying transversely between digestive glands and intestine. × 8.

Fig. 13. Lateral view of *Mo 17* showing nearly normal development of digestive organs following duplication of the first third of the midgut. × 7.

Fig. 14. Ventral view of *Mo' 8*, a specimen exhibiting nearly complete regulation following duplication of the first half of the mesenteron. × 7.

Fig. 15. Lateral view of *Mo' 34*. *p.*, primary pancreas; *p'.*, secondary pancreas. × 7.

Fig. 16. Ventrolateral view of *Mo' 13* showing an extra pancreatic duct, *p.d.* × 7.

Fig. 17. Lateral view of *Mo'' 3*, an example in which the first two thirds of the midgut was duplicated. *p.*, primary pancreas; *p'.*, secondary pancreas; *s.*, primary stomach; *s'.*, secondary stomach. × 7.

Fig. 18. Lateral view of *Mi 2* dissected to show the slight enlargement in the second turn of the outer intestinal coil. × 7.

Fig. 19. Lateral view of *LivRev 12*, an example showing nearly complete regulation after rotation of the first third of floor of the midgut. × 7.

Fig. 20. Ventral view of *LivRev 4* showing the stomach, *s.*, and pancreas, *p.*, displaced to the left and separated widely from the liver, *l.*; the intestine, *i.*, joins the stomach anteriorly on the left side of the coelom. × 7.

Fig. 21. Ventral view of *LivRev 19*. *l.*, liver; *p.*, pancreas; *s.*, stomach; *i.*, intestine; *g.b.*, gall bladder. × 7.

Fig. 22. Ventral view of *IntRev 30*, an example in which the coiling of the intestine became irregular following rotation of the middle third of the mesenteron. × 7.

Fig. 23. Ventral view of *IntRev 23* showing reduced coiling and enlarged diameter of the intestine. × 7.

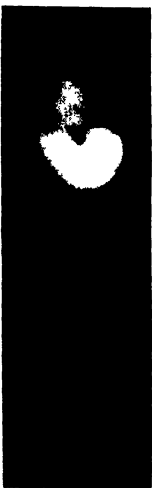Fig. 24. Ventral view of *IntRev 21*, an example showing almost complete regulation. × 7.

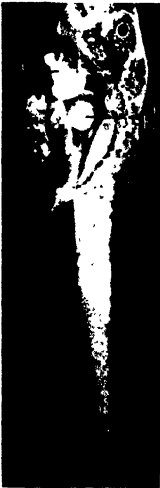[182]

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AN EXPERIMENTAL STUDY OF THE HISTOLOGICAL AND FUNCTIONAL DIFFERENTIATION OF THE EPITHELIAL HYPOPHYSIS IN HYLA REGILLA

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INTRODUCTION

THE NORMAL embryology of the pituitary body, involving the approximation and fusion of originally separate components, suggests that one or both differentiates correlatively with respect to the other. The first experimental evidence concerning the relationship was obtained by Philip Smith (1916) in connection with hypophysectomy of young frog larvae. He suggested that the pars neuralis of the pituitary required association with the epithelial pars buccalis in order to reach full development and observed (1920) that an atypically placed epithelial hypophysis may cause hypertrophy of adjacent neural tissue. The discovery by Holt (1921) of a 40 mm. pig foetus in which the pars neuralis had developed normally in the absence of an epithelial hypophysis, however, tended to refute Smith's view.

Stein (1929) undertook to determine the capacities for independent differentiation of the chick pars buccalis and infundibulum by means of chorio-allantoic grafts. Interdependence of the two components seemed to be indicated but the evidence was inconclusive. Blount (1930, 1932) tested the developmental capacities of the pars buccalis of *Amblystoma punctatum* by homoioplastic transplantation The epithelial anlage was removed from the stomodeal region shortly after it appeared and was transplanted heterotopically, both with and without adjacent neural tissue. Examination of the resulting larvae revealed that those grafts which included neural tissue had differentiated in a typical manner, whereas grafts of the pars buccalis alone had failed to do so. It was inferred that the pars buccalis differentiated correlatively with respect to adjacent brain tissue.

Atwell and Holley (1936) extirpated the caudal part of the epithelial hypophysis of tailbud embryos of *Rana sylvatica*. The albinism characteristic of intermediate-lobe-deficiency appeared, and histological examination of the experimental animals revealed complete absence of the pars intermedia. In accounting for the absence of this lobe Atwell suggested two possibilities: (1) the caudal part of the pars buccalis alone contained the potentiality for forming pars intermedia; and (2) the rostral part of the hypophysis, if equipotent for pars intermedia, could not form an intermediate lobe owing to the interference with normal contact between stomodeal and neural components of the pituitary body.

Other experiments by Atwell (1937, 1940) and those of Etkin (1935, 1940) led these investigators to the conclusion that the pars buccalis is independ-

ently differentiating. The work of both supported this view for *Rana sylvatica* and *R. pipiens,* and that of Atwell (1940) indicated that it is true of *Amblystoma jeffersonianum, A. tigrinum,* and the partially albino strain of *A. mexicanum.* Atwell (1935, 1937) used *A. punctatum* also, but obtained no unequivocal case of differentiation and function of the transplanted hypophysis, a circumstance which bears out the work of Blount on this form. Etkin (1941, 1943) has suggested that the hypophysis may be dependent upon neural tissue for its "pre-primordial" development but after reaching its definitive position in the embryo it is capable of independent differentiation. An analysis of the several investigations of the development of the amphibian pituitary will be made later in this study.

Bearing upon the problem are the experiments of Gaillard (1937), who cultivated explants of the pars anterior of a three-month-old rabbit together with explants of pars nervosa, thyroid gland, adrenal gland, and other organs. He found that those parts of the anterior lobe which were adjacent to the explant of pars nervosa gave rise to an "intermediate-lobe-like" structure. Gaillard suggested the possibility that the partes intermedia and tuberalis are differentiated from the pars buccalis under the direct influence of the pars neuralis and the infundibular body, respectively.

The inferences of Blount and Gaillard were borne out in part by experiments which involved shifting the presumptive hypothalamic part of the brain caudad from its normal position in the late gastrula stage of *Hyla regilla* (Burch, 1938). The pars buccalis, when it eventually formed and took up its definitive position, was thus unable to make contact with the pars neuralis. The pars intermedia failed to differentiate in the isolated pars buccalis of the experimental animals, and the pigmentary conditions characteristic of its absence appeared. From further work (Burch, 1939) it appeared that the pars buccalis likewise failed to differentiate into a functional pars anterior. It was suggested that the infundibular region exercises an inductive influence upon the pars buccalis, which is transmitted through contact, and is necessary for the normal differentiation of the epithelial hypophysis. This paper presents more complete evidence for this opinion.

MATERIALS AND METHODS

All experiments to be described were performed upon embryos of the Pacific tree frog, *Hyla regilla.* The developmental ages of the embryos are designated according to a series of stages in the normal development of *Hyla regilla* (Eakin, 1946), the number and features of the stages being similar to those given by Shumway (1940) for *Rana pipiens.*

Microsurgical operations were performed by means of microscalpels (Burch, 1942), glass needles, and other instruments commonly used in experimental amphibian embryology. Although rigidly sterile conditions were not maintained, aseptic techniques were employed as much as possible. Growing larvae were kept in pond water and fed maximally with cooked, strained spinach and egg yolk.

Histological methods included the use of Bouin's and Zenker-formal fixa-

tives; and haematoxylin-eosin, and Van Giesen's stains. For cytological studies of the pituitary body a modification of the Mallory-azan staining technique was employed.

EXPERIMENTS

The capacity of the pars buccalis for self-determination was tested by two types of experiments. The immediate purpose of the first experiment was so to alter the developing embryo of *Hyla regilla* that the pars buccalis and pars neuralis of the pituitary body would, when formed, retain their integrity but never come into relationship with each other. This was accomplished by moving the presumptive pars neuralis out of its normal position at an early period in development. The translocation was performed at the very late gastrula stage when the medullary plate was first indicated by faint lines of pigment. The location of the presumptive pars neuralis had been determined in previous experiments by means of vital staining technique. For purposes of comparison, normal and hypophysectomized embryos of the same age as the experimental animals were maintained under comparable conditions.

A second series of experiments, similar to those of other investigators, involved the complete removal and heterotopic, autoplastic transplantation of the pars buccalis at a stage prior to its differentiation. These operations were performed upon tailbud embryos of *Hyla regilla* for the purpose of comparing the reactions in this species with those reported for other forms and of determining when, if at all, the pars buccalis becomes capable of continuing its development alone.

Experiment 1. Caudal Translocation of the Presumptive Pars Neuralis

The presumptive infundibular region of the brain in the late gastrula stage of *Hyla regilla* (stage 12+) occupies a small, median, dorsal area, which lies approximately at the junction of the anterior and middle thirds of the embryo. An area containing this anlage in its anterior part was excised from the dorsal wall of the gastrula, turned end for end, and replaced in the site from which it was taken (fig. A). The longitudinal axis of the graft was thus reversed and the anlage of the infundibular region was shifted somewhat caudally.

In a series of 104 such operations, there was no immediate mortality. Late mortality was, however, high. This was owing, in part, to the occasional presence of defects in the buccal apparatus, which interfered with feeding. The buccal defects usually consisted in reduction of the size of the mouth and immobility of the jaws. The cause of these abnormalities is not known, since the operations did not involve the presumptive buccal area. The majority of the tadpoles, however, survived for periods ranging between two and four weeks, in which time inanition, fungous infection, and death from other causes resulted in the loss of about 80 per cent of the specimens. The remainder of the tadpoles were hardy and free of deformities. Of these, 22 survived the normal larval period, as judged from the metamorphosis of the normal controls.

Experimental tadpoles from the early operations of the series frequently exhibited anomalies of the eyes (pl. 17, fig. 2). These usually amounted to

no more than a slight reduction in size, but in several instances only small vesicles remained. The incidence of such defects subsided altogether when the operation was performed at slightly more posterior levels. The source of these anomalies was the injury to the eye anlagen which resulted when the operation was incorrectly done (Spemann, 1906).

Of the 104 experimental tadpoles, 89 exhibited the albinism which characterizes lack of the pars intermedia of the pituitary body, whereas 15 were normally pigmented. After the loss of 82 specimens, in the course of three weeks, there remained 18 albino and 4 normally pigmented experimental larvae which were maintained for periods ranging from 34 to 108 days. One albino and the 4 normally pigmented specimens, with controls, were fixed about one month after operation. A second group of 11 albino tadpoles was from 40 to 58 days old and a third group of 6 albinos, whose controls had metamorphosed, was 65 to 108 days old when fixed for histological study. Normal controls required from 65 to 80 days for metamorphosis under laboratory conditions.
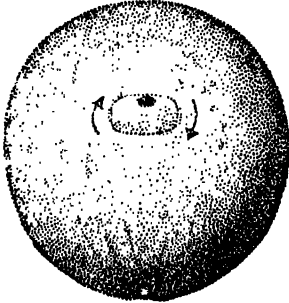
Fig. A. Dorsal view of late gastrula (stage 12+) of *Hyla regilla*, showing position of presumptive infundibulum (dark spot) with reference to the piece which was rotated through 180°.

The albino tadpoles (pl. 17, figs. 2 and 4) exhibited the complete pigmentary anomaly described by Smith (1916), Allen (1917), and Atwell (1921a). The superficial melanophores were smaller and fewer in number than in the normal condition and nonchromatophoric melanin in the epidermis was reduced. The most conspicuous change, however, was in the dermal melanophores, in which the melanosomes had become maximally aggregated. The pigment of the xantholeucophores, on the other hand, was well dispersed. It was possible to reverse the distribution of the pigment in the two types of chromatophores by injecting an aqueous extract of the adult pituitary body of *Hyla* into the subcutaneous spaces of the albino tadpole. The reversal took place within fifteen minutes.

Following a 4- to 6-week period of rapid growth, the experimental tadpoles and the normal and hypophysectomized controls tended to assume a nearly uniform and constant size. Variation in size among the experimental tadpoles was slightly greater than in the normal controls but they were, on the whole, as large as the latter. The average size of the hypophysectomized controls was less than that of the former groups, although some specimens were fully as large as any of the normal tadpoles. It was frequently observed that the smaller individuals of the hypophysectomized group had defects of the buccal region, which doubtless interfered with feeding.

The albino experimental tadpoles, with one exception, showed no signs of progress toward metamorphosis. Minute, translucent hindlimb buds made their appearance somewhat later than in the normal tadpoles, but failed to differentiate or to show any growth disproportionate to that of the body. In the exceptional instance, a considerable degree of differentiation of the thigh,

lower leg, and digits occurred (pl. 17, fig. 4). This was not accompanied by the usual acceleration in growth seen at approaching metamorphosis.

Serial, longitudinal sections of two of the normally pigmented experimental larvae revealed that the operation to shift the infundibulum had not been made at the proper level in the presumptive medullary plate. The major part of the infundibulum was in its normal position and the epithelial hypophysis, having come into association with it, was normally differentiated. In all the albino tadpoles, on the other hand, it was found that with some variation in degree, the infundibulum had, indeed, been shifted caudally from its normal
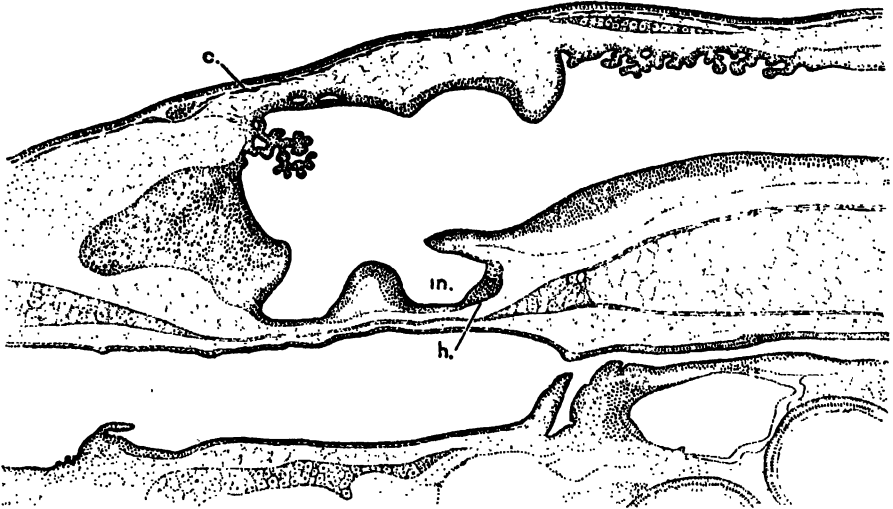
Fig. B. Sagittal section through a normal tadpole of *Hyla regilla. c.*, dermal chromatophores showing expanded melanosomes; *h.*, epithelial hypophysis, differentiated into anterior and intermediate lobes, situated along posteroventral floor of infundibulum; *in.*, infundibulum.

position in the posteroventral wall of the diencephalon (*in.*, fig. B) and was appended to the rhombencephalon (*in.*, fig. C). There was occasionally a slight development of the infundibulum in the normal position but this was usually insufficient to extend below the general level of the brain floor. The shape of the transposed infundibulum was frequently distorted but was always represented by a pronounced ventral extension of the fourth ventricle. In those specimens in which the infundibulum was relatively undistorted, its orientation was obviously reversed, the thin ependymal wall being situated anteriorly (fig. C).

The pars buccalis in the albino tadpole appeared to have been formed in the normal manner from the deep ectodermal cells of the embryonic stomodeal region and to have assumed its definitive position beneath the diencephalon in the larva. In the normal tadpole the hypophysis (*h.*, fig. B) would have become affixed to the posteroventral aspect of the infundibulum. In the albino experimental tadpole, however, this relationship was not attained and the pars buccalis remained as an isolated mass of cells (*h.*, fig. C). In some of the specimens the pars buccalis was partly or entirely enclosed by cartilage,

derived from the cranial floor, being thus effectively insulated from contact with neural tissue (pl. 18, fig. 5). In others, however, the pars buccalis had unobstructed access to neural tissue, including that of the infundibulum, but had not made contact (see, for example, pl. 18, fig. 6).

The normal epithelial hypophysis of the month-old tadpole of *Hyla regilla* lies posterior and ventral to the infundibulum, the contiguous wall of which gives rise to the pars nervosa of the pituitary body. The hypophysis consists of three morphologically and histologically distinct parts: the pars anterior, pars intermedia, and pars tuberalis. The pars anterior and pars intermedia (*a.*, and *i.*, respectively, pl. 18, fig. 7) correspond well to descriptions of these premetamorphic structures in species of *Rana* (D'Angelo, 1941). Three types
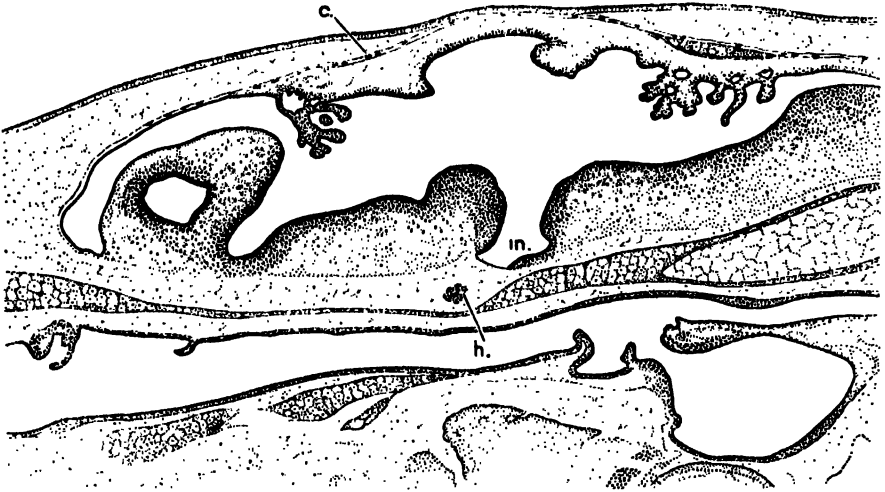
Fig. C. Sagittal section through an experimental albino tadpole of *Hyla regilla*. *c.*, dermal chromatophores showing contracted melanosomes; *h.*, isolated and undifferentiated epithelial hypophysis; *in.*, translocated infundibulum, seen appended to floor of rhombencephalon.

of cells and possibly a fourth are distinguishable in the pars anterior (pl. 18, fig. 8). These are, in the order of decreasing abundance: (1) medium-sized cells (9–11μ) with indistinct cell boundaries and sparse, fine, slightly acidophilic granules; (2) large cells (12–15μ) with distinct boundaries and densely packed, strongly acidophilic granules; (3) large cells (13–16μ) with distinct boundaries and nongranular, basophilic cytoplasm. The possible fourth type of cell is smaller than the first, and contains very few granules, which are faintly acidophilic or colorless. Intergrades between this and the first type of cell are to be found. All types of cells have oval, vesicular nuclei with one to several nucleoli. The pars intermedia lies immediately posterior to the pars anterior. Its cells are large (15–18μ), polyhedral, and of nearly uniform size. The nuclei are vesicular and the finely granular cytoplasm is slightly basophilic.

The isolated pars buccalis of the experimental larva is composed entirely of cells (pl. 18, fig. 9) of nearly uniform size and tinctorial reaction, and correspond well with those of the fourth type described for the normal gland.

The cells are relatively small (8–10μ) and their boundaries are not distinct. The cytoplasm contains very fine, sparse granules, which are faintly acidophilic. A few large, irregular granules of melanin occur in some of the cells. On the whole, the cells are smaller and less granular than those of type 1 in the normal gland. The nuclei, which are irregularly oval and contain one or more nucleoli, stain intensely and tend to obscure the cytoplasmic picture in sections thicker than 6μ. The cells are frequently arranged in rosettes around a central space, giving the whole structure an acinar appearance (pl. 18, fig. 5). Reference will be made to this characteristic later. The cells may, however, be packed together with no apparent order. Occasional blood vessels occur in or near the structure but it is not highly vascular. It is enclosed in an extremely delicate capsule of connective tissue. No differentiation into lobes occurred in any of the albino experimental tadpoles.

Evidence regarding the functional state of the isolated pars buccalis was sought in comparative studies of the thyroid glands and gonads of the experimental tadpoles and their normal and hypophysectomized controls. The studies were made near the end of the larval period, a time when the activity of the normal pituitary gland is at a high level.

The thyroid gland of the normal two-month-old *Hyla regilla* tadpole consists of two well-separated lobes, situated in the hypobranchial region. Each lobe (pl. 19, figs. 10 and 12) is elongate and oval and contains from 12 to 18 follicles, of various sizes and shapes, filled with a vacuolated colloid. The cells of the follicular epithelium are columnar. The basally placed nuclei are vesicular and the cytoplasm contains fine, slightly acidophilic granules, small vacuoles and numerous, filamentous mitochondria around the nuclei. The boundaries of the cells are distinct and their apices, frequently irregular, bulge into the lumen of the follicle. The gland is very vascular and, as judged by the foregoing criteria, is highly active.

In the experimental albino tadpole of the same age and size, the thyroid gland is considerably smaller and contains fewer and smaller follicles in which colloid is present (pl. 19, figs. 11 and 13). The most conspicuous difference between the experimental and the normal gland, however, is seen in the character of the follicular epithelium, which, in the experimental tadpoles is cuboidal and scarcely exceeds the height of the nuclei (pl. 19, fig. 11). The nuclei are smaller than in the normal gland and the scant cytoplasm contains few granules, no vacuoles, and no mitochondria. Intercellular boundaries are indistinct and the apices of the cells are smooth and regular. The blood supply of the gland is less well developed than that of the normal animal.

The foregoing description holds, in its essentials, for the thyroid gland of the hypophysectomized tadpole (pl. 19, fig. 14). The gland in the hypophysectomized controls is, in the main, slightly smaller than that of the experimental tadpoles of corresponding age. In some control specimens, where incomplete hypophysectomy had left a small fraction of the epithelial hypophysis, the thyroid gland showed evidence of much greater activity than in the experimental tadpoles.

Examination of the gonads of the experimental and control tadpoles yielded

no evidence regarding the gonadotropic activity of the isolated pars buccalis. The gonads were in comparable stages of development in the experimental, the normal, and hypophysectomized tadpoles of similar age and size. The larval gonad and that of the newly metamorphosed adult do not reflect the activity of the pituitary body in a degree sufficient for estimation of its functional state.

No attempt was made to use the adrenal gland as a means of assessing the activity of the pituitary body.

EXPERIMENT 2. TRANSPLANTATION OF THE EMBRYONIC PARS BUCCALIS

The pars buccalis of the tailbud embryo of the anuran amphibian originates as a medial proliferation from the deep ectodermal cells about the stomodeal region. This flattened cone of cells, broadly connected anteriorly, projects inward between the roof of the foregut and the floor of the prosencephalon and is in close contact with both.

Exposure of the structure for transplantation was accomplished by cutting the stomodeal area frontally, in order to reveal the roof of the foregut. This epithelium was then stripped off and the brain floor and pars buccalis brought into view. The epithelial hypophysis was removed intact by loosening its posterior extremity from the brain floor with the convex side of a fine glass hook, which was then passed gently forward between the pars buccalis and the wall of the brain. The hypophysis was freed from connection with the anterior deep ectoderm by means of a microscalpel or glass stylet. Extreme care was taken to remove all vestiges of the pars buccalis since previous experience had revealed the striking powers of regeneration of the structure. Equal care was taken not to include any of the tissue of the floor of the brain.

Having been removed from its orthotopic position, the pars buccalis was implanted just behind the pronephros, which is manifest externally at this stage as a lateral bulge posterior to the region of the gills. Healing at the site of the transplant and in the buccal region was rapid and there was at first no appreciable mortality in a large number of such operations. Later, inanition, as a result of buccal defects, caused the loss of a small percentage of such specimens and a somewhat larger number died from other causes. The majority, however, survived for a period of 15 to 45 days, when they were fixed for histological study.

Preliminary experiments with a variety of developmental stages in the tailbud range had indicated a variability in the capacity of the pars buccals to differentiate normally as a transplant. On the basis of these experiments three age groups (stages 18+, 19–, and 19) were selected for critical investigation of the differentiating capacity of the transplanted pars buccalis. The operations upon the three groups were identical. Both normal and hypophysectomized control specimens were kept for each of the groups.

The youngest age group (stage 18+) included 10 experimental animals which had been operated upon prior to contact between the pars buccalis and the floor of the infundibulum (fig. D). Of the 10 specimens, 8 showed approximately the same lack of pigmentation and retardation in growth as the

hypophysectomized controls. The remaining 2 tadpoles were pigmented in a degree approaching the normal control and showed normal growth and progress toward metamorphosis. Results of the preliminary experiments led to the belief that the two normally pigmented tadpoles retained some orthotopic pituitary tissue and that this, rather than the transplanted gland, was responsible for maintenance of the normal condition. A second operation was, therefore, performed upon these two specimens. This operation involved the removal and retransplantation of the grafts from the pigmented tadpoles into totally albino specimens. Both operations were successful. The transplants, together with a segment of the mesonephric duct and some muscle,

survived in their new position under the dorsal skin of the albino hosts, and retained the structure characteristic of autoplastic, heterotopic transplants. The transplants, however, did not exert any melanophorotropic activity, either generally or locally. The belief that they were not responsible for melanosome expansion in the original hosts was further substantiated when orthotopic pituitary was found in those animals upon sectioning them.

Of the 8 albino tadpoles, 5 were sectioned serially and stained for histological study. All were found to be entirely free of orthotopic pituitary tissue and the transplants were identified in all specimens. The morphology of the transplanted pars buccalis in

Fig. D. Sagittal section of the head of a tailbud embryo (stage 18+) of *Hyla regilla*. Epithelial hypophysis (*h.*) has not made contact with infundibulum (*in.*).

this age group of tadpoles is very characteristic. It takes the form, usually, of a single large follicle (pl. 20, figs. 15 and 16), although two and sometimes three such structures occur. The walls of the follicles are composed in some instances of cuboidal or squamous cells, and in others of columnar cells. In the latter, the apices of the cells are directed toward the cavity. Within the cavity is an intensely basophilic substance, resembling colloid of the thyroid, which is apparently secreted by the cells, the cytoplasm of which becomes increasingly basophilic toward the apex.

The intermediate group of embryos (stage 19–) exhibited the degree of development of the pars buccalis illustrated by the sagittal section in figure E. Transplantation of the structure was accomplished with greater facility in this group than in the preceding one because of its greater size and discreteness. The operation was performed upon embryos of two different spawnings, which were, as nearly as could be determined, in the same developmental stage. A total of 28 transplantations was made and all specimens survived the operation for 3 to 6 weeks, the majority of the group being fixed at approximately 30 days of age.

The appearance of the month-old tadpoles was not uniform. Of the 28 specimens, 9 were albino, 6 were normally pigmented, and 13 ranged between albinism and the normal condition. Two of the last group displayed localized expansion of the melanosomes in the region of the graft but were otherwise albino. The intermediate character of the largest group was due primarily to submaximal expansion of the dermal melanosomes. Although the melanophores also appeared to be fewer in number, no counts were made to confirm this impression. As in Experiment 1 (translocation of the infundibular
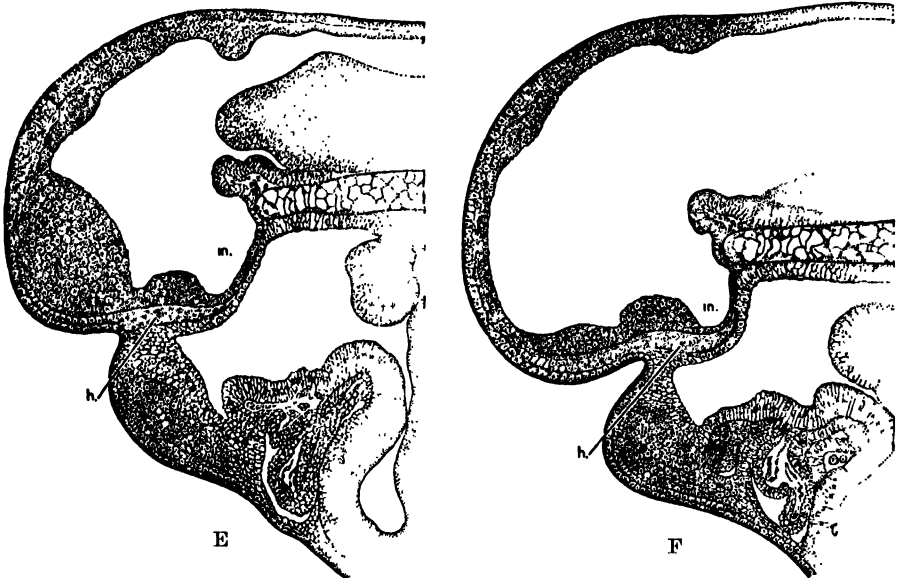
Fig. E. Sagittal section of the head of a tailbud embryo (stage 19–) of *Hyla regilla.* Epithelial hypophysis (*h.*) is bordering on anterior margin of infundibulum (*in.*).

Fig. F. Sagittal section of the head of a tailbud embryo (stage 19) of *Hyla regilla.* Epithelial hypophysis (*h.*) is in contact with floor of infundibulum (*in.*).

anlage), the experimental tadpoles as well as hypophysectomized and normal controls exhibited variation in size which bore no specific relationship to the operative procedure.

Of the entire group of 28 experimental tadpoles, 15 were sectioned serially and stained for histological study. Among these were 4 albinos, 5 normally pigmented tadpoles, and 6 specimens showing intermediate pigmentation including those with local expansion of melanosomes. All the histological preparations were carefully examined for orthotopic pituitary tissue and for the transplanted pars buccalis.

The findings may be summarized as follows:

1) Four albino tadpoles: Orthotopic pituitary tissue completely absent in three; a trace present in one. Large, follicular transplants present in three; small nonfollicular transplant present in one.

2) Five normally pigmented tadpoles: Orthotopic pituitary present in all. Follicular implant present in four; nonfollicular implant present in one.

3) Six tadpoles showing intermediate pigmentation: Orthotopic pituitary tissue pres-

ent in large amount in one; merest trace present in two; completely absent in three, including two specimens which showed localized pigmentation (pl. 4, fig. 17). Follicular implants present in five; small, nonfollicular implant present in the one with large orthotopic pituitary.

In the oldest group of experimental animals (stage 19) the developing pituitary was in the stage illustrated in figure F, with the epithelial hypophysis in direct contact with the thin floor of the infundibulum. The complete extirpation of the pars buccalis was easily performed at this stage. The group contained seven experimental animals, all of them normally pigmented. They were sacrificed at three weeks and sectioned serially. Upon microscopic examination, it was found that the epithelial hypophysis, which had been transplanted autoplastically to the region of the pronephros, had differentiated morphologically and histologically into lobes resembling the pars anterior and the pars intermedia of the normal control. Compare figure 18 (pl. 20), a photomicrograph of one of the transplants in this age group, with figure 7 (pl. 18), a section through the pituitary gland of a normal tadpole. No hypophyseal tissue was found in the orthotopic position. It is to be noted that ganglion cells seen in figure 18 were traced along a large nerve to an adjacent dorsal root ganglion. Other specimens did not show ganglion cells, and differentiation of these cells should not be construed as the result of the inadvertent inclusion of nervous tissue with the graft.

DISCUSSION

The results of the experiments reported here indicate that in *Hyla regilla* contact between the epithelial hypophysis and neural tissue, specifically infundibulum, is necessary for differentiation and function of the intermediate and anterior lobes of the pituitary body. In the reconciliation of these results with those of Atwell (1935, 1936, 1937, 1940) and Etkin (1935, 1938, 1940, 1941, 1943) three possibilities should be considered.

First, the differences in results obtained by varous workers may well be attributed to differences inherent in different species of Amphibia. It has been learned through studies on other problems, such as the induction of the amphibian lens, that developmental processes are not always the same even within a group of closely related organisms (Spemann, 1938). The experiments here reported, especially those involving the caudal displacement of the infundibular rudiment, should be repeated on other amphibian types, particularly *Rana sylvatica* and *R. pipiens,* upon which Atwell and Etkin performed many of their experiments.

A second possibility is that in some of the previous experiments, neural tissue, especially that of the infundibulum, was inadvertently included with the transplanted primordium of the hypophysis and was responsible for its differentiation and function. Atwell (1937) used precautions not to include neural tissue with his transplants, but in 3 of the 4 "successful" experimental specimens of *Amblystoma punctatum* neural tissue was subsequently found associated with the graft and was believed by Atwell to have been carried with the transplant from the infundibular region. Most recently, Etkin (1941,

1943) transplanted the hypophyseal rudiment in several species of anurans with as little adherent tissue as possible and found many of the grafts to be entirely free of neural tissue when the specimens were sectioned and examined. This renders unlikely the possibility that the differentiation and function of Etkin's hypophyseal grafts was due to the association of neural tissue after transplantation. It should be noted that the method for disrupting the association of the neural and epithelial components of the pituitary body, which was described in the first part of this paper, is carried out in the late gastrula when these components are far apart, thus permitting operative procedures on one without injury to or inclusions of the other. The problem of painstaking dissection of the hypophysis in the tailbud stage and the uncertainty about its freedom from neural elements therefore does not arise.

A third possible explanation of the apparent discrepancy between the results and conclusions of Atwell and Etkin and those reported here, might assume that contact between epithelial hypophysis and infundibular floor had already been effected before these investigators performed their transplantations. In this event, the differentiation of the heterotopic pars buccalis might be due to an inductive influence already exerted by the floor of the brain upon the pars buccalis before the latter was transplanted. The phenomenon of evocation is known to require only a short period of contact between inductor and substratum in other instances.

Atwell and Etkin have not specified the precise developmental age of the anuran embryos used in their experiments. Their stages were described as tailbud embryos. The tailbud stage, however, embraces a considerable range of relationships between the pars buccalis and the infundibulum (see figs. D–F). In a relatively few hours a tailbud embryo of *Hyla regilla* develops from a stage (fig. E) in which the caudal tip of the hypophyseal anlage borders on the diencephalic outpocketing to a stage (fig. F) in which the pars buccalis is definitely in contact with half the infundibular floor. It seems not improbable that these authors used anuran embryos in which contact of the neural and epithelial rudiments had already occurred. If so, the embryos were too old for a critical analysis of the problem.

Although the developmental ages of the urodeles which have been used in transplantation experiments have been given more precisely by reference to Harrison's series of amphibian developmental stages, uncertainty about the exact relationships of the epithelial and neural components of the pituitary body still exists. It is possible that a single stage of Harrison's series may encompass a significant range of relationships between these components for any one species and quite probably that variations of relationship occur in different species in the same stage of the series. Embryos corresponding approximately to stages 30 to 32 of Harrison's series for *Amblystoma punctatum* were used by Atwell in his experiments upon this and several other species of *Amblystoma*. From such descriptions and figures as are available (Atwell, 1921*b* and Blount, 1932) it would appear that in *Amblystoma punctatum* stages 31 and 32 of Harrison's series have the pars buccalis bordering on or in actual contact with the infundibulum. Embryos in which such re-

lationships exist at the time of operation would seem to be too old and their use productive of misleading results.

It is to be observed here that a large proportion of Atwell's transplantations were considered unsuccessful in the sense that the graft did not function as a pituitary body. The interpretation of results was apparently based only upon the "successful" operations in which the material may have been too old at the time of operation. Atwell (1937, 1940) obtained 35 functional transplants of the hypophysis in *Amblystoma jeffersonianum*, 3 in *A. tigrinum*, 2 in *A. mexicanum*, and 4 in *A. punctatum*. The number of operations performed upon each of the first three species is not known, but it is assumed that the per cent of functional transplants was highest in *A. jeffersonianum*. The success with this species may have been owing to a more rapid relative development of its pituitary body. It is also possible that more of the embryos of *A. jeffersonianum* were in stage 32 when the operations were performed.

Blount (1932) working on embryos of *Amblystoma punctatum* in stages 23 to 31, obtained no functional transplants of the hypophysis when the grafts were free of neural tissue. He states, "... the stages used in these experiments include those before the hypophysis shows visible development and in no case is it more than an epithelial thickening" (p. 115). The fact that Atwell and Blount have reported opposite results with the same species when the major difference in experimental conditions seems to have consisted only in the ages of the embryos used, lends support to the third explanation for differences between Atwell's and Etkin's results and conclusions and those reported here.

In experiments reported earlier (Burch, 1938, 1939) and in detail in the first part of this paper, the infundibulum rather than the epithelial hypophysis was transposed to an heterotopic site. This procedure, carried out in the late gastrula, before either the brain or the pars buccalis is formed, allows the hypophyseal anlage to fulfill its intrinsic developmental capacity without ever coming into close relationship with the infundibulum. Under these circumstances, the hypophyseal anlage is formed normally from the deep ectoderm of the stomodeal region, grows inward beneath the prosencephalon as a tongue of cells, becomes constricted from its connection with the stomodeal ectoderm, and comes to lie in its normal site, as an isolated body between the diencephalon and the floor of the developing cranium. It is of interest that the pars buccalis grows inward to a certain extent and moves no farther. Instances have been found where the pars buccalis was separated from the infundibular floor by a fraction of a millimeter only, with no barrier whatsoever to association. It would seem that there is no attraction between these components but that their association is brought about solely through the mechanical processes of growth.

The essential advantage of the experimental method just described is that it permits the disruption of the potential association of the neural and epithelial components at an early stage rather than the separation of the nearly or completely associated components at a comparatively late stage of development. Although the results of the method indicate that the pars buccalis

differentiates correlatively with respect to the infundibulum, the experiment provides no information on the time requirements for the association.

The second series of experiments was planned to confirm the findings of the first series, to ascertain the minimum of association of the components of the pituitary body necessary for its complete differentiation, and to test the third possible explanation for the conflicting reports described above. Three critical stages in the development of the pituitary body of *Hyla regilla* (figs. D–F) were selected as donors of hypophyseal transplants. Heterotopic, autoplastic transplantations of the pars buccalis in stage 18+ (fig. D), when hypophysis and infundibulum are not yet in contact, yielded no instance of a functional pars intermedia. Transplantation performed in stage 19– (fig. E), when the hypophyseal anlage is just bordering on the infundibulum, resulted in some silvery and some pigmented tadpoles whereas all embryos upon which the operation was performed in stage 19 (fig. F), when the pars buccalis was definitely in contact with the infundibular floor, exhibited normal pigmentation and the graft showed both pars intermedia and pars anterior.

Of special interest were two albino specimens of the intermediate age group (19–) in which localized pigmentation occurred in the skin overlying the transplant (pl. 20, fig. 17). There was no observable differentiation of a pars intermedia. That the entire transplant underwent incomplete differentiation with partial function as a result of insufficient association with the infundibulum seems improbable. It would seem more likely that a few of the cells of the transplant underwent complete differentiation or that the localized pigmentary effect was nonspecific.

This series of experiments thus seems to confirm the finding in the first series. It furthermore indicates that contact between the epithelial hypophysis and the infundibulum needs to exist only a short time in *Hyla regilla* to enable differentiation and function of the pars intermedia. No evidence respecting the function of pars anterior was sought in this series of experiments. Bearing in mind that the present experiments were performed upon a different family and in some cases a different order of Amphibia, the results lend further support to the view that the opposed reports which have appeared in the literature have been due to the use of embryos of different ages; specifically, that the reports of independent differentiation of the hypophysis have been based upon experiments with embryos which were too old.

It is emphasized that the findings and conclusions reported here hold strictly only for *Hyla regilla* and although the general principles may and probably do hold for other forms, certain variations, especially in time relations are to be expected. In this connection three reports are of interest. Hegre (1942) has found that "epithelial rudiments alone from stage 33 of *Amblystoma maculatum (punctatum)* gave rise to no pars intermedia (103 cases)." Blount's experiments (1939), relating to the time of determination of the pars anterior, have seemed to show that embryos, from which the brain was removed before any thickening of the hypophyseal rudiment occurred (stage 24), developed a poorly differentiated pars anterior, capable of some thyrotropic activity. There was no development of a pars intermedia. Full

accounts of these investigations have not yet been published.[1] Eakin (1939) reported an instance of an albino larva of *Triturus torosus* in which the hypophysis and infundibulum had been separated by internal pressure resulting from the injection of gelatin into the embryonic foregut. The silvery condition of the tadpole and the histological findings suggested that in *T. torosus* establishment and maintenance of contact between pars buccalis and infundibulum may be necessary for normal function of the pars intermedia. At the time the operation was performed buccal and neural primordia were undoubtedly in contact with each other. The albinism, however, might have been due to factors peculiar to the experimental procedure which was used.

In speaking of the differentiation of the pars buccalis three phases may be distinguished in *Hyla regilla*: the early phase, prior to contact with the infundibular area; a second phase, when through contact with the infundibulum an impetus to full development is obtained; and a final phase, in which full development is reached. Although there is no experimental proof of functional differentiation of the pars buccalis in the first phase, the growth pattern and cellular morphology justify the belief that the early pars buccalis is undergoing change preparatory to its final state. Atwell (1936) accepted this view and made it the basis of an experiment on the development of the pars intermedia.

The mechanism of the second phase, which may be called the inductive phase, remains conjectural, as do those of several better recognized instances of induction such as evocation of the medullary plate and the lens. The infundibular area alone has the ability to induce the pars buccalis; this power seems to be lacking in parts of the brain adjacent to the infundibulum. Certain features distinguish the infundibular area and may play the essential role. Among these is the character of its walls when the "ingrowth" of the epithelial hypophysis occurs (see fig. F). At this time the wall consists only of ependymal cells. In structures adjacent to the infundibulum, on the other hand, the ependymal layer is insulated by the mantle and marginal layers. It will be recalled that almost from the beginning of its "ingrowth" the epithelial hypophysis is in contact with neural tissue anterior to the presumptive infundibulum. Failure to obtain functional heterotopic transplants of the early hypophyseal primordium, as shown by Blount (1932) and certain of the experiments reported here, might be due to the primary absence of an evocator in neural tissue other than the infundibulum or to the insulation of the ependymal cells by the mantle and marginal layers of the brain. The inductive capacity of the ependymal cells other than those of the infundibulum might be tested by implants of the early pars buccalis into the neurocoel. It seems certain that the influence of contact is not exerted by nervous mechanisms in the commonly accepted sense. A humoral or biotactic phenomenon seems most probable. Whatever the mechanism, the inductive phase seems to

[1] Blount's paper (1945) appeared when this paper was in press. Unfortunately it cannot be considered at length here. There is very good agreement between his findings and those reported here with respect to the determination of the pars intermedia, but not with respect to the histological and functional differentiation of the pars anterior.

be essential for the final phase of development of the hypophysis in *Hyla regilla*.

The final, postinductive phase in the development of the hypophysis in *Hyla regilla* is characterized by marked growth and differentiation of the pars buccalis into the functional hypophysis of the tadpole. This phase does not require contact with the infundibulum or other neural tissue for its continuance. It may be regarded simply as the fulfillment of a potentiality, initiated in the inductive phase.

It is of interest, both in the present connection and with respect to the problem of induction by the infundibulum, that Etkin (1941, 1943) has shown that the normal growth and function of the differentiated pars intermedia of *Rana* is dependent upon the maintenance of contact of this lobe with the infundibulum. If this relationship is broken by transplantation of the definitive hypophysis without infundibular tissue, the pars intermedia exhibits hyperplasia and hyperfunction, as shown by intensely pigmented experimental tadpoles.

SUMMARY

1. In the first series of experiments the presumptive infundibulum of the brain was translocated caudally to the region of the future hindbrain by a 180° rotation of a piece of the presumptive medullary plate in very late gastrulae (stage 12+) of the Pacific tree frog, *Hyla regilla*.

2. The experimental animals of this series exhibited the following features in the tadpole stage:

a) An albinism, identical with that produced by hypophysectomy, in which superficial melanophores were smaller and fewer, nonchromatophoric melanin in the epidermis was reduced, dermal melanosomes were contracted, and the pigment of xantholeucophores was dispersed.

b) Little or no progress toward metamorphosis.

c) No infundibulum associated with the diencephalon but instead a reversed infundibular depression in the floor of the rhombencephalon.

d) An isolated, morphologically undifferentiated, vesicle-like hypophysis which contained none of the cellular types characteristic of the normal gland such as acidophils and basophils, but consisted of small chromophobic cells.

e) A hypoplastic thyroid, similar to that found in hypophysectomized animals, in which follicles were few and small and the follicular epithelium was low-cuboidal or squamous.

3. In a second series of experiments, a graded series of tailbud embryos of *Hyla regilla* (stages 18+, 19−, and 19) were used as donors and hosts for autoplastic, heterotopic transplantation of the epithelial hypophysis.

4. The experimental animals, in which the orthotopic hypophysis was completely removed and transplanted exhibited the following features:

a) Experimental animals, in which the transplantation was performed in stage 18+ (epithelial hypophysis not in contact with infundibulum), were silvery like the hypophysectomized controls and also the experimental animals of the first series. The transplants remained undifferentiated vesicle-like masses of cells.

. *b*) Experimental animals, in which the transplantation was performed in stage 19 (epithelial hypophysis in contact with infundibulum), were normally pigmented. The grafts were found to be differentiated into pars anterior and pars intermedia.

c) Experimental animals, in which the transplantation was performed in stage 19– (epithelial hypophysis bordering on anterior margin of infundibulum), showed a condition of pigmentation intermediate between normal and hypophysectomized controls. The transplants were in some instances follicular, in some instances, nonfollicular.

5. It is concluded that contact between pars buccalis and nervous tissue, specifically infundibulum, is necessary for the histological and functional differentiation of the epithelial hypophysis. Without this contact cellular types such as acidophils and basophils do not develop and intermedin and thyrotropic hormone are not produced, as indicated respectively by the albinism and the hypoplastic thyroid.

6. The results and conclusion of this paper are compared with the findings and conclusions of Atwell, Blount, Etkin, and others.

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Fig. 1. Young normal tadpole of *Hyla regilla*. Note the expanded melanosomes, the black tapetum of the eye, and the condensed yellow lipochrome of the xantholeucophores. This tadpole was a control of the same age as the experimental animal shown in figure 2.

Fig. 2. Young experimental tadpole of *Hyla regilla* showing the silvery condition resulting from the caudal translocation of the presumptive pars neuralis of the late gastrula. Compared with the control (fig. 1), it can be observed that in this specimen the superficial melanophores are smaller and fewer in number, that nonchromatophoric melanin in the skin is reduced, and that the melanosomes of dermal melanophores are maximally concentrated, whereas the pigment of the xantholeucophores is well dispersed. This specimen was selected from a group of imperfect experimentals which showed defects of eye and mouth (see above, p. 188).

Fig. 3. Normal, almost completely metamorphosed tadpole of *Hyla regilla*. This animal was a control to the experimental tadpole shown in figure 4.

Fig. 4. Experimental tadpole of *Hyla regilla* 108 days following the operation in which the presumptive pars neuralis of the late gastrula was translocated posteriorly. This animal, a representative of the group of experimentals showing complete absence of buccal and optic defects, exhibits the albinism which results from the above operation. Note especially the silvery eye; dispersed pigment in the xantholeucophores completely obscures the black tapetum. This specimen was the only example which showed any progress toward metamorphosis (see above, p. 188). Note the partly differentiated hindlimb; compare, however, with the control of the same age shown in figure 3.
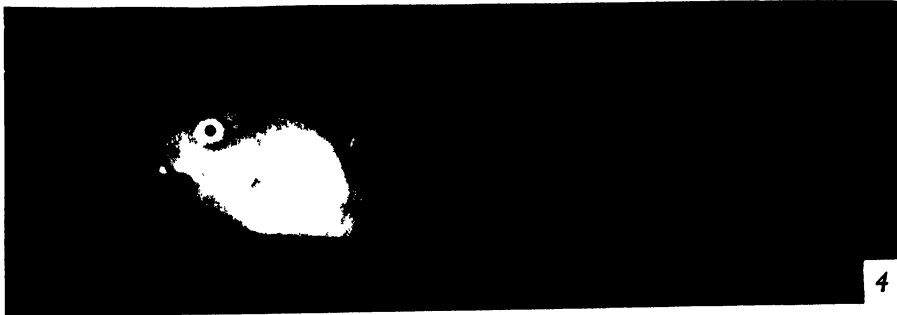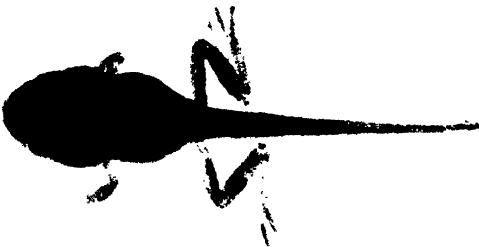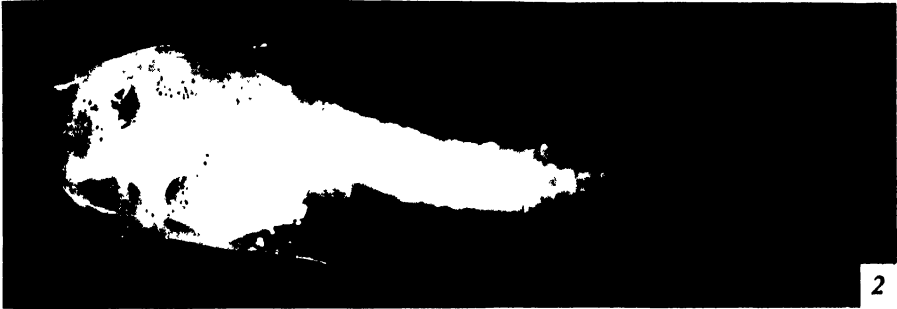
PLATE 18
(Photomicrographs)

Fig. 5. The isolated pars buccalis of an albino tadpole of *Hyla regilla* in which the presumptive infundibulum had been shifted caudad in the late gastrula stage. The anatomical position of the isolated hypophysis is shown in text figure C. Note that the pars buccalis, enclosed by cartilage, was effectively insulated from contact with neural tissue. Its undifferentiated cells are arranged in cords about central spaces, giving the whole structure the appearance of a vesicle. Compare with the normal hypophysis shown in figure 7.

Fig. 6. Another example of an isolated pars buccalis of an albino tadpole. In this instance the pars buccalis had an unobstructed access to neural tissue (*nt.*) with which it had apparently not made close contact. The infundibulum, normally developing at this level (see text figure B and figure 7), had been shifted caudad in the late gastrula stage.

Fig. 7. The pituitary gland of a normal tadpole of *Hyla regilla*. The normal epithelial hypophysis lies posterior and ventral to the infundibulum (*in.*), the contiguous wall of which forms the pars nervosa of the pituitary body. Note the dark pars anterior (*a.*), the smaller and lighter pars intermedia (*i.*).

Fig. 8. High magnification of the pars anterior of the normal epithelial hypophysis shown in figure 7. Note: *c.*, chromophobes, medium-sized cells $(9-11\mu)$ with sparse, slightly acidophilic granules; *a.*, acidophils, large cells $(12-15\mu)$ with distinct boundaries and densely packed, strongly acidophilic granules; $\beta.$, a basophil, a large cell $(13-16\mu)$ with distinct boundaries and nongranular, basophilic cytoplasm.

Fig. 9. High magnification of the isolated epithelial hypophysis shown in figure 5. Note that the cells possess nearly uniform size and staining reaction. Acidophils and basophils found in the normal hypophysis (see fig. 8) are not differentiated. The cells here are relatively small $(8-10\mu)$; cellular boundaries are indistinct; the cytoplasm contains very fine, sparse granules which are slightly acidophilic. Compare with chromophobes of the normal gland.
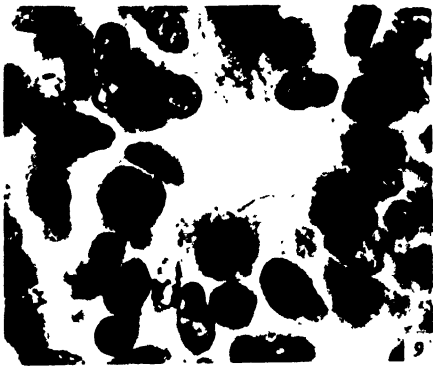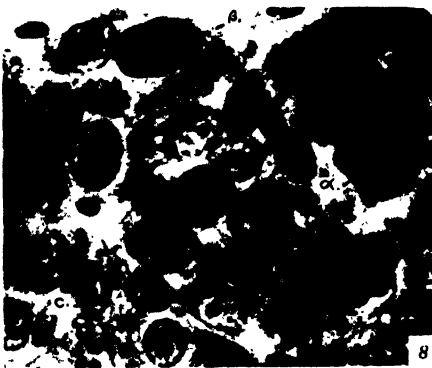
PLATE 19
(Photomicrographs)

Fig. 10. Section through the thyroid gland of a young normal tadpole of *Hyla regilla.*

Fig. 11. Section through the thyroid gland of an experimental tadpole of *Hyla regilla* in which the presumptive infundibulum was translocated, in the gastrula stage, to the region of the future hindbrain. Note the small size of the gland, the small number and size of the follicles, and especially the low, almost squamous, epithelium. Compare with the control of the same age shown in figure 10.

Fig. 12. Section through the thyroid gland of a normal tadpole of *Hyla regilla* just before metamorphosis. Note the large size of the gland, the large number and size of the follicles, the high columnar epithelium, and the vacuolated colloid.

Fig. 13. Section through the thyroid gland of another example of an experimental tadpole. Compare with the control of the same age shown in figure 12.

Fig. 14. Section through the thyroid gland of a hypophysectomized tadpole of *Hyla regilla*. Note the low-cuboidal epithelium. Compare with the normal gland (fig. 12) and with the thyroid in the experimental animal (fig. 13) in height of epithelium.

Figs. 15 and 16. Sections through the pars buccalis which had been auto-
plastically and heterotopically transplanted in embryos of *Hyla regilla* in stage
18+, in which hypophysis and infundibulum had not yet made contact. Note
that the graft has formed a vesicle-like structure; pars anterior and pars inter-
media cannot be identified morphologically or histologically.

Fig. 17. Section through the pars buccalis which had been autoplastically and
heterotopically transplanted in an embryo of *Hyla regilla* in stage 19–, in which
the hypophysis was bordering on the anterior margin of the infundibulum. The
transplant consists largely of three small follicles. Note that the melanosomes
(*m.*) of near-by dermal melanophores are expanded; elsewhere in the tadpole
they were contracted.

Fig. 18. Section through the pars buccalis of *Hyla regilla* which has differen-
tiated from the autoplastically and heterotopically transplanted epithelial
hypophysis. The operation was made in an embryo in stage 19, in which contact
between pars buccalis and infundibulum had been established. Both pars
anterior (*a.*) and pars intermedia (*i.*) may be identified. Compare with the
normal pituitary (fig. 7).

THE ROLE OF THE BASIBRANCHIAL CARTILAGES IN THE EARLY DEVELOPMENT OF THE THYROID OF HYLA REGILLA

BY

MIRIAM STOKES JAMES

CONTENTS

THE ROLE OF THE BASIBRANCHIAL CARTILAGES IN THE EARLY DEVELOPMENT OF THE THYROID OF HYLA REGILLA

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MIRIAM STOKES JAMES

INTRODUCTION

THE ORIGIN of the amphibian thyroid as a ventral outgrowth from the floor of the foregut has been seen in many species since Müller (1871) first demonstrated the course of its development in *Rana temporaria*. Subsequent investigations have traced further the elongation of the single thyroid bud and its division into two parts which migrate caudad as the definitive larval glands. During the entire embryonic development of the thyroid, as well as in the adult position, the gland is closely related to the cartilages which constitute the hyobranchial apparatus. The formation of those cartilages in the Anura has been described by Parker (1871) and by Gaupp (1906). D'Angelo and Charipper (1939) traced the movement of the thyroid relative to the cartilages, but they made no suggestion about a developmental relationship between the two structures. Gasche (1939), in a study of the urodele thyroid, has indicated that the "pendulum" shape characteristic of the anlage is produced by the formation of the hyoid cartilages on either side of the thyroid bud, which constrict it. An experimental analysis of development in the two structures, one entodermal and one mesodermal, has suggested a developmental relationship, purely physical in nature, between them.

Müller (1871) and other early investigators noted that the amphibian thyroid, in its migration caudad, was apparently split into two lobes by the copula, or basihyal-basibranchial portion of the hyobranchial apparatus. Allen (1930) noted, in transplants of larval thyroids from *Rana aurora* grafted to older larvae, the occasional formation of cartilages from "mesenchymal material" which had been grafted along with the thyroid anlage. In a single example a basibranchial cartilage, with its characteristic keel, was formed, and in that graft the thyroid divided to form two lobes, one on each side of the cartilage. In another instance, where a cartilaginous rod was formed, the gland enveloped it, but was not divided. Gasche (1939) noted the division of the thyroid, but did not suggest that a mechanical factor might be involved.

The origin of the major part of the hyobranchial apparatus from cells of the neural crest, or mesectoderm, is significant for this aspect of thyroid morphogenesis. Landacre (1921) and Stone (1922) first noted the migration of neural crest cells into the cranial region, and observed that those cells could be easily distinguished from the mesentodermal cells (i.e., the mesoderm of the visceral arches) by their pigment and their smaller yolk platelets. Stone (1926, 1932), by a series of extirpations of the neural crest in *Amblystoma*,

has shown that the entire hyobranchial skeleton, except for the second basi-branchium which arises entirely from mesentoderm, is formed from mesecto-derm superimposed upon a core of mesentoderm. Moreover, mesectoderm transplanted to heterotopic positions gives rise to cartilage, whereas trans-planted mesentoderm produces muscle, connective tissue, and blood vessels, but no cartilage *except* the second basibranchium (Stone, 1932). Similar experiments on *Rana palustris* (Stone, 1929) have shown that the same holds for the anuran embryo.

The present investigation demonstrates, through extirpation and trans-plantation of embryonic tissues, the relation of the hyobranchial apparatus, and in particular the second basibranchium, to the morphogenesis of the anuran thyroid. The work was carried on under the direction of Professor J. Frank Daniel, for whose guidance and helpful criticism the author is most grateful.

MATERIAL AND METHODS

Embryos of the Pacific tree frog, *Hyla regilla,* were used as material for experimental study. They were collected in the vicinity of Berkeley, Califor-nia, and previous to operation were kept in a small artificial pond or in aquaria in the laboratory.

For the most part, standard procedures of experimental embryology were employed. Wide-mouthed pipettes served for transferring the eggs, and hair-loops were used in manipulating them. For the actual operative procedure very fine needles were found most suitable. They were ground down to ex-tremely fine points on a small Arkansas stone under a dissecting microscope and were fastened into pin vises for use.

All experimental animals were maintained after operation in small dishes of pond water and were fed, after yolk resorption, on canned, strained spinach. The preparation of animals for study varied according to the experi-ment, as did the operative procedure, and the techniques involved will there-fore be included in the description of individual experiments.

EXPERIMENTAL PROCEDURE

NORMAL DEVELOPMENT OF THE THYROID IN RELATION TO THE HYOBRANCHIAL APPARATUS OF *Hyla regilla*

In a 4.5 mm. embryo of *Hyla regilla* the thyroid, which in the 2 mm. animal arose as a knotlike projection from the floor of the pharynx, has elongated until its distal end is flattened against the pericardium. At 5 mm., a frontal section shows a separation of the distal end of the anlage into two halves. Between the two halves lies the second basibranchial portion of the hyo-branchial apparatus (*bb–2,* pl. 21, fig. 1), identified, according to Stone (1922), by its larger yolk platelets. Dissection at this stage reveals the thyroid (*th.,* fig. A, 1) as a pigmented mass, single anteriorly but divided into two lobes posteriorly and dorsally, and closely applied to the second basibranchium. Further gross development consists of the complete separation of the two
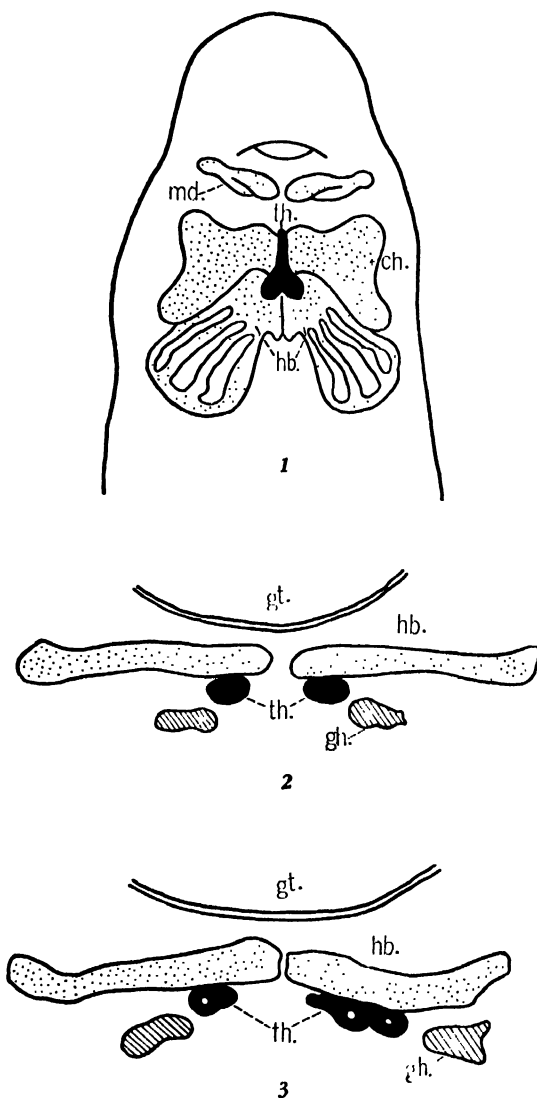
Fig. A. Diagrams of the later development of the thyroid in *Hyla regilla*. 1, ventral view of hyobranchial apparatus in 7 mm. larva; 2, cross section through region of thyroid, 7 mm. larva; 3, same, 10 mm. larva. *ch.*, ceratohyal cartilage; *gt.*, foregut; *hb.*, hypobranchial cartilage; *th.*, thyroid; *md.*, mandibular arch; *gh.*, geniohyoid muscle.

halves of the gland from the floor of the foregut and their migration caudad
to their definitive positions. In a 7 mm. larva they lie median and dorsal to
the geniohyoid muscles and ventral to the hypobranchial portion of the hyoid
apparatus (*th.,* fig. A, 2).

Structure and Development of Hyobranchial Apparatus in *Hyla regilla*

The hyobranchial cartilages of *Hyla regilla* are similar to those described
for *Rana* by Stone and earlier investigators, except that in *Hyla* the basihyal
is absent. The cartilages, formed by the migration of neural crest cells ven-
trally over the core of mesentoderm, are foreshadowed in sections of a 4 mm.
embryo by more dense masses of cells in the loose mesenchyme of the head
region. As the stomodaeum breaks through, the hyoid arches, directly pos-
terior to the mandibular arches, fuse to form the wings of the cerato-hyoid
division (*ch.,* fig. B, 2). From their point of fusion, called the *pars reunians* by
early investigators, a tongue of cells grows caudad as the first basibranchium
(*bb-1*). Posteriorly the four branchial arches (*br.,* fig. B, 2) have formed and
their proximal divisions unite to produce the thin, flat plates of the hypo-
branchial cartilages (*hb.*), which fuse in the midline. When the fusion of the
elements is complete and the cartilaginous matrix is being laid down, the
second basibranchium (*bb-2*, fig. B, 2) may be seen in sections as a distinct
knob of cells, loaded with yolk platelets, just posterior to the first basibranch-
ium. On this knob, in a 5 mm. larva, the thyroid appears to divide (pl. 21,
fig. 1). Later the deposition within it of a cartilaginous matrix results in the
formation of a distinct keel-like projection; and the second basibranchium,
although closely joined to the first, can still be distinguished as a separate
structure.

Experiments Demonstrating a Relation Between the Thyroid and the Second Basibranchial Cartilage

Three types of operation were performed to determine the role of the basi-
branchial cartilages in the division of the thyroid. Varied attempts were made
to secure embryos with hyobranchial cartilages complete *except for* the second
basibranchium by removing the median ventral mesentoderm before the ven-
tral fusion of the mesectodermal components.

EXTIRPATION OF VENTRAL MESODERM ALONE

Embryos were selected in which the neural folds were high and close together,
but not fused. An embryo was placed, ventral side up, in an operating dish,
a flap of ectoderm and mesoderm was peeled away from an area just posterior
to the future stomodaeum, and an attempt was made to remove all mesodermal
cells from the region. With a hairloop the mesodermal cells were scraped from
the ectoderm of the flap, and mesodermal cells still adhering to the thin ento-
derm of the foregut were removed with fine needles. The ectodermal flap alone
was then replaced and held down with a bridge until healing was complete.

The larvae were sacrificed 12 days after operation, fixed in 95 per cent

alcohol, and dissected. Those to be studied further were stained *in toto* with toluidin blue (Williams, 1940). After destaining in alcohol they were stored in methyl salicylate and were studied in that fluid or in alcohol. In a few instances the cartilages were dissected out and mounted on slides for closer study or for camera lucida drawings.
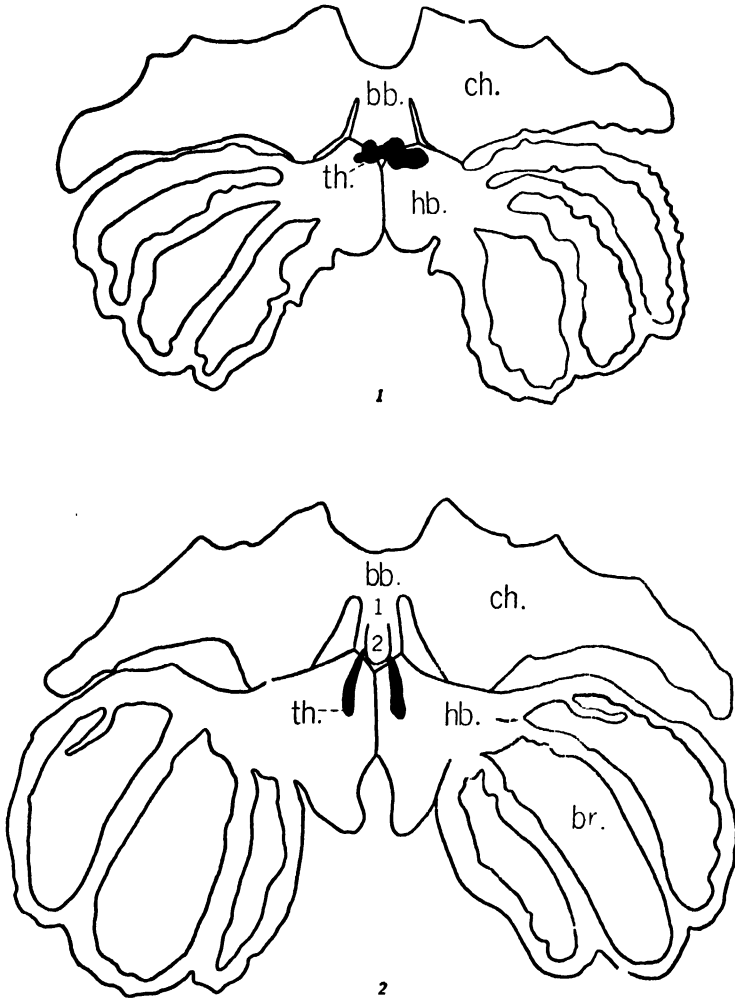
Fig. B. Hyobranchial apparatus from 15-day larva of *Hyla regilla*. 1, larva from which anterior mesentoderm has been removed; 2, normal control; *bb.*, basibranchial; *br.*, branchials; *ch.*, ceratohyal; *hb.*, hypobranchial; *th.*, thyroid. Camera luc.da drawings, ventral view.

Fifty-four operations were performed, and 44 animals were available for study. Of these, 12 possessed single, median thyroids. Study of the cartilages of these specimens revealed that in every instance the second basibranchium was absent (fig. B, 1). The first basibranchium appeared as a flat plate to the posterior end of which the hypobranchial cartilages were attached, but there was no evidence of the typical keeled appearance (see fig. B, 2) of the normal

copula. In the remaining animals complete regeneration of mesentoderm had apparently taken place. The mesentodermal cells which give rise to the second basibranchium are very close to the entoderm, and it is difficult to remove all of them without injury to the thin floor of the foregut. A few cells left in place seem to be sufficient to regenerate the whole of the missing basibranchium.

EXTIRPATION OF VENTRAL ECTODERM-MESODERM, AND REPLACEMENT BY FOREIGN ECTODERM-MESODERM

Pairs of embryos were selected in which the neural folds had just joined along their entire length. At a similar stage in *Rana palustris,* according to Stone (1929), the neural crest material, in its migration from the neural tube, is still high on the sides of the body. The same holds true for *Hyla regilla.* A rectangle of ectoderm extending from the center of the sensory plate, back for about one-fifth the length of the body, and laterally across the entire ventral surface, was removed from one of each pair of embryos, and the underlying mesoderm was carefully cleaned away from the entoderm until the entire area was judged free from mesodermal cells. An equivalent area of ectoderm, plus its underlying mesoderm, was taken from the posterior ventral region of its companion embryo, just anterior to the blastopore, and was placed over the exposed entoderm. A bridge held the graft in place until healing was completed. Animals were sacrificed at 15–20 days after operation, and were fixed in Bouin's fluid. The mortality was high, owing to the extensive nature of the operation, but 38 animals were sectioned and studied.

Fifteen animals were secured in which the entire copula (first and second basibranchials) was missing. The absence of the first basibranchium probably indicates that the broad strip of mesoderm which had been removed included the ventral edge of the migrating mesectoderm, the area which gives rise to the first basibranchium. Each of these 15 animals possessed a single, median thyroid. In 3 specimens the gland was still connected with the floor of the foregut (pl. 21, fig. 2), and in several others the point of attachment was indicated by a strand of cells. Ten of the 38 animals were found to lack only the second basibranchium. Of these, 8 had single, rounded thyroids, whereas 2 had single, broad and flat glands stretching across the ventral surface of the first basibranchium (pl. 21, fig. 3). In addition, in several larvae the regenerated basibrachium was abnormal in position, that is, it was tipped far to one side, toward the hypobranchial cartilage. Five animals possessed such distorted cartilages, and all had single, median thyroids.

In all instances where heart development and circulation were normal, the single thyroid was histologically indistinguishable from the normal paired thyroids of the control animals (pl. 21, fig. 4).

EXTIRPATION OF ALL THREE GERM LAYERS

In certain individuals from an earlier series of attempted thyroid extirpations, where all three germ layers were removed from the floor of the foregut, the entire anlage of the thyroid was not removed, and thyroid glands developed. Since the median ventral mesoderm just posterior to the mouth

had been extirpated, deficiencies in the hyobranchial cartilages frequently occured. In 5 such animals the entire copula was absent, and in all of these a single thyroid was observed. Nine animals lacked only the second basibranchium. In 8 of these the thyroid was a single, median structure, whereas the one remaining animal possessed a diffuse, flattened gland applied to the surface of the first basibranchium.

The evidence from the three series of experiments indicates that the division of the thyroid into the two lobes characteristic of the amphibian is due to the mechanical effect of the keel-like second basibranchium, which splits the anlage longitudinally, and that simultaneous elongation of the thyroid bud and ventral extension of the developing cartilaginous knob or keel are the factors involved.

DISCUSSION

This study indicates that the division of the thyroid anlage distally is due to mechanical pressure from the keel-like second basibranchium. Certainly the thyroid, in the process of division, is seen to be applied to its ventral surface. Gasche (1939) has suggested, for *Salamandra*, that the "pendulum" shape whi h is characteristic of the thyroid bud at this stage is the result of the simultaneous formation, laterally, of the two halves of the hyobranchial apparatus, which compress the proximal part of the anlage. His explanation probably holds true also for *Hyla regilla*, since in the 4–5 mm. embryo the dense masses which comprise the primordia of these structures can be seen on the sides of the developing thyroid. As the thyroid moves caudad, the halves of the future hyoid (ceratohyoid) cartilages fuse, dorsal to the anlage. The first basibranchium, which is formed by proliferation from their point of union, is only slightly convex in cross section, so that the thyroid moves posteriorly under an almost plane surface. Later in development, according to Stone (1929) and according to observations in the present investigation, the second basibranchium is formed, so that the thyroid, in its growth caudad, meets the prominent, median knob of cells. Division of the gland begins posteriorly and dorsally, and continues through the entire distal part of the mass, which by this time has severed its connection with the foregut. The two halves move down on either side of the second basibranchium and across the flat surfaces of the hypobranchial cartilages to their definitive positions. In the absence of the second basibranchium the entire anlage moves caudad as a single mass, which flattens out across the first basibranchium, later rounding up as follicles begin to develop.

In a series of grafts of th thyroid anlage into older embryos, made at the same time as the experiments described above, there was no evidence for the division of the thyroid by the basibranchial cartilage, as was described by Allen (1930) for a single specimen. Allen's transplants were made at a later stage, when the thyroid, in its migration caudad, was very close to the developing cartilage. In the present series, any presumptive cartilage included in the graft lay posterior to the anlage of the thyroid. Since the external physical factors in the normal environment of the thyroid were not present (the grafts were placed in the pronephric region), an approximation of the

two structures was not possible; and in no instance, among the experimental animals, was cartilage seen in the immediate vicinity of thyroid follicles.

The elongation of the thyroid bud and its extension caudad toward the heart cannot yet be fully explained, but a suggestion can be made concerning the factors involved. It must be remembered that, simultaneously with the formation and elongation of the thyroid bud, the entire foregut is changing by rapid growth, at a rate greater, in fact, than that of its surroundings. From a short, undifferentiated region of the archenteron, it becomes a relatively elongate structure, narrower at the anterior end, broad and flat in the vicinity of the pharynx. Most of the growth occurs in the region not impeded by yolk, that is, in the region anterior to and immediately surrounding the thyroid. It is conceivable that during this rapid elongation of the foregut the physical forces thereby created bring about the change in the relative position of the thyroid. Evidence suggestive of the action of physical forces, such as tension, upon the developing thyroid appears in the flat, elongate shape of the young glands, and in the tendency of the cells to become oriented in longitudinal strands.

The fusion of the two tubular components of the heart in the ventral midline occurs at the same time as the caudad migration of the thyroid anlage; and the elongation, as well as the subsequent coiling of that organ, may also be involved in the extension of the thyroid. The rapid growth of the foregut and the heart is the usually accepted cause for the posterior migration of the mammalian thyroid and thymus (Kingsbury, 1915), and it may well be a factor in the development of the amphibian thyroid.

It is possible, then, to demonstrate experimentally the relation of the anuran thyroid to the copular portion of the hyobranchial apparatus, a relation described by certain of the early investigators of amphibian development. The structure involved, however, appears to be not the entire copula, but only the keel-like second basibranchium, which is formed later than the other components and lies directly in the path of the rapidly elongating thyroid. Since an undivided thyroid often forms when the second basibranchium is "out of line" as well as when it is absent, it is probable that the relationship between thyroid and copula is not associated with any morphogenetic substance, but is the result of purely physical forces produced by differential growth.

SUMMARY AND CONCLUSIONS

1. The development of the thyroid gland in relation to the hyobranchial apparatus has been traced in the larva of *Hyla regilla.*

2. By removing the ventral mesentoderm of the visceral arches, previous to the downward migration of the neural crest cells (mesectoderm), larvae have been obtained in which the hyobranchial apparatus is complete except for the second basibranchium. The latter is mesentodermal in origin, and arises later than the other cartilages.

3. Animals which lack the entire copula, and animals which lack only the second basibranchium, possess single, median thyroids. When only the second basibranchium is missing, the thyroid lies close against the flat ventral side

of the first basibranchium. When both cartilages are missing, a connection with the pharynx may be maintained.

4. The presence of the keel-shaped second basibranchium directly in the path of the migrating thyroid bud is evidently the cause for the division of the gland. The elongation of the thyroid bud, as a result of growth and mechanical stress, and the ventral extension of the knoblike cartilage, occurring simultaneously, seem to be the factors involved.

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PLATE

PLATE 21

Fig. 1. Cross section through the developing hyobranchial apparatus of a 5-mm. embryo, showing the dividing thyroid (*th.*) closely applied to the second basibranchium (*bb–2*).

Fig. 2. Cross section through a larva from which the anterior ventral mesoderm has been removed and replaced by posterior mesoderm. Neither basibranchial cartilage is present, and the single, median thyroid (*th.*) has maintained its connection with the floor of the pharynx; *hb.*, by hypobranchial cartilage.

Fig. 3. Cross section through a larva from the same series, in which only the second basibranchium is missing. Note the broad, flat thyroid (*th.*) ventral to the first basibranchium (*bb–1*); *ch.*, ceratohyal cartilage.

Fig. 4. Cross section through an older larva from which the anterior ventral mesoderm was removed and replaced by posterior mesoderm. Only the second basibranchium is absent. Normal follicles are present in the single thyroid (*th.*) which lies lateral to the slightly tilted first basibranchium (*bb–1*).

QUALITATIVE VARIATION OF THE HYPOPHYSEAL THYROTROPIC HORMONE IN THE VERTEBRATES

BY

AUBREY GORBMAN

CONTENTS

QUALITATIVE VARIATION OF THE HYPO-
PHYSEAL THYROTROPIC HORMONE
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INTRODUCTION

EVER SINCE Houssay (1929) made the suggestion that a "zoological specificity" might exist in certain of the hormones of the pituitary, this possibility has been the object of considerable interest. Cumulative data gained in work with the thyrotropic hormone, as well as with other hypophyseal factors, generally indicate that these agents are able to achieve their typical effects when administered to some other species, or even class of animals [reviews and bibliographies in Witschi (1937); Gorbman (1941); Adams and Allen (1942)]. In the light of such evidence it would seem that a concept of strict "zoological specificity," as originally suggested by Houssay, which would require that the thryotropic hormone have no activity in an alien species or class, is not tenable. There remains for careful consideration, however, a real possibility that certain minor interspecific hormonal differences may affect significantly the efficiency of action of any given thryotropin when tested in another species. In addition to its more academic interest, during the past decade this idea has frequently concerned endocrinologists in choosing animal species to be used either as subjects for the bioassay of hypophyseal hormones or as sources of pituitary material for extraction. Certainly some differences in quantity of hypophyseal hormones produced may be expected, and have been claimed, among vertebrate species, but what of qualitative differences? Chemical data are now available indicating that the hormones of the anterior pituitary are proteins, and that there are demonstrable slight differences in these proteins between species. The immunological evidence gathered in investigating the problem of hypophyseal antihormones would seem to support this view (Gordon, 1937). The chemical evidence presented by Li, Lyons, and Evans (1940, 1941) seems to establish this point of view conclusively. These workers found definite differences in solubility, electrophoretic behavior, and in amino acid content between "pure" lactogenic hormones of cattle and of sheep. Similar specific differences, as well as variation in molecular weight, were found between gonadotropic hormones (interstitial cell stimulating factor) of sheep and pigs (Li, Simpson, and Evans, 1942).

The existence of chemical specificities in hypophyseal hormones of different species may be regarded as reasonably well confirmed. On the other hand, the physiological value of such chemical variations is not easily determined. To test this point the experiments reported here were designed especially to find whether thyrotropic proteins have a variable activity when assayed in animals

of different species. Since this variability could be demonstrated only on a comparative basis the following procedure was developed. It was proposed at the outset to assay carefully a series of pituitary thyrotropic preparations, obtained from several different vertebrate classes, in a series of test animals, the recipients representing, in turn, several classes of vertebrates. It could be anticipated that in any given type of test animal each of the thyrotropic preparations would have a definite and determinate degree of activity or potency, and that for that test animal a list of the tested thyrotropic preparations could be made, and arranged in the order of decreasing potency. If for all other species that were subjects of assays, the order of relative potencies of the thyrotropins were the same, then it could be assumed that all the thyrotropins behave the same physiologically, regardless of chemically demonstrable protein differences. If the relative potency in different test animals is not the same, then it is manifest that the chemical differences in protein thyrotropins, minor though they may be, do have physiological value, since thyroids of different test animals can "discriminate" between thyrotropins, reacting relatively more strongly to some, and less strongly to others.

When the difficulties involved in a project necessitating careful reciprocal bioassays of pituitary hormones of higher and lower vertebrates are realized it is not surprising that this problem has not attracted many investigators. For example, because the pituitaries of most lower vertebrates are small, thousands of animals must be dissected to obtain an adequate supply of pituitary glands for a complete study. To add to the difficulty, in many vertebrates the hypophyseal target organs have a seasonal cycle of activity, making it necessary to complete all experiments within a short time.

MATERIALS AND METHODS

Thyrotropins were tested in four kinds of animals, representing four different vertebrate classes. Seven pituitary preparations, taken from four of the vertebrate classes, were used as the sources of thyrotropic hormone.

Animals used for testing.—Small, adult goldfish uniformly two inches long, obtained from a local dealer, were kept in a healthy, vigorous condition in groups of five in one-gallon jars, and were fed a prepared fish food.

As the amphibian test animal, slender salamanders (*Batrachoseps attenuatus*) were collected on the day before they were first used. In the rainy season, these animals occur in large numbers under rocks, logs, and other debris at Berkeley, California. They were conveniently kept in groups of five, in large covered paper cups containing a pad of moistened paper toweling. They were not fed in the period of treatment since they carried adequate amounts of stored fat in the large fat-bodies.

Fence lizards (*Sceloporus occidentalis*) were collected in Strawberry Canyon in the eastern part of the campus of the University of California in the summer and early autumn. They were kept in a large outdoor cage and were

fed meal worms until the middle of November, when they began hibernation by burying themselves in the soft earth and under boards and rocks in the cage. In the first week in January the lizards were dug out of the ground for use in the laboratory, and were all, therefore, in the hibernating stage at the time of injection.

Young guinea pigs, weighing from 200 to 240 grams, were selected as the mammalian test animals. These were kept in cages in a room at a constant temperature of 82° F. during the period of injection.

Pituitary preparations used.—Fish pituitaries were collected on board trawlers working out of San Francisco and Fort Bragg, California. It was not practicable to store glands from different species separately, but all pituitaries were from fish known on the commercial market as "sole" and belonging to the family *Pleuronectidae*. Almost the entire collection was obtained from three species: *Atheresthes stomias*, the arrow-toothed sole; *Perophrys vetulus*, the pointed-nosed sole; and *Eopsetta jordani*, the round-nosed sole. The glands were removed from the head within an hour after capture and placed in a large volume of acetone which was changed twice before the glands were powdered. In the laboratory the glands were dried, ground to a powder, weighed, and stored in a desiccator in the refrigerator until used. Conditions prevailing at the time of collection made it impossible to count the number of glands obtained, and therefore to compute their average weight. An amount slightly in excess of five grams of dried pituitary was obtained; as nearly as can be estimated, this represents about 3,000 glands.

For amphibian pituitaries two species of frogs were used. Heads of one of these, *Rana pipiens*, the leopard frog, were obtained from a commercial establishment in Chicago. The heads had been removed from the carcasses within an hour after death and placed in a large volume of acetone, which was stirred and changed once. The heads were then shipped to Berkeley, where more than 2,500 pituitaries were dissected out, dried, powdered, and weighed. About 800 pituitaries of the bull frog, *Rana catesbeiana*, were similarly obtained at a commercial establishment in San Francisco. The pituitary powders of both prepared from the acetone-dried glands was stored in a desiccator in a refrigerator until the powders were used.

Pituitaries of chickens were collected in insulated boxes charged with solid carbon dioxide ("dry ice"). Heads were removed from freshly killed chickens, placed in a freezing box, and frozen solid within two hours following death. They were thawed within twenty-four hours and cut parasagittally on a power jig saw to expose the hypophyses. More than 2,300 hypophyses were so removed and were placed immediately in acetone. The pituitary powder prepared from the acetone-dried glands was stored in a desiccator in a refrigerator.

Three different preparations of mammalian pituitary were used. A crude powder prepared by precipitation of a 40 per cent alcohol extract of sheep pituitary (powder no. IID28B) was kindly supplied by Dr. H. M. Evans of the Institute of Experimental Biology of the University of California. A relatively purified preparation of thyrotropic hormone derived from pituitaries of sheep and pigs (powder no. Th 9) was furnished by Dr. A. S. Parkes.

As yet, there is no "international standard" thyrotropic preparation, but a personal communication from Dr. Parkes states that the Th 9 powder, which contains four Parkes-Rowlands thyrotropic units per milligram, will be equated to the standard when it becomes available.

Method of treatment.—Saline solution suspensions of all powders were prepared on the day preceding the first injection, and were kept frozen when not actually in use. The suspensions were administered intraperitoneally daily for five days and the animals were autopsied on the sixth day. For the guinea pigs, injections were given daily for three days only, with autopsy on the fourth day. This schedule was observed in order to adhere to the usual method of assay in guinea pigs. Because of differences in size among the test animals, the dosage was varied in each case. The individual doses for each animal are listed in tables 1, 2, 3, and 4. For a given hormone three levels of dosage usually were established. A unit group of five (salamander, fish) or four (lizard, guinea pig) test animals received a given dosage level of any particular hormone. In addition, a few animals were given unusually high doses of each hormone preparation. For the guinea pigs daily determinations of oxygen consumption were made in a Benedict multiple unit machine as a check of the thyrotropic activity of each preparation.

For each type of test animal used control animals were injected with Ringer's solution; the doses are given in tables 1–4.

All tissues were fixed in Bouin's fluid and embedded in a rubber-beeswax-paraffin mixture. Serial sections were cut at 10μ and stained in Mallory's triple connective-tissue stain, or iron-haematoxylin, aniline blue, methyl green stain, as recommended by Koneff (1936). The most consistent criterion of thyroid stimulation was height of follicle cells. Other criteria, also used, were vacuolization of colloid, uniformity in staining properties of colloid, vascularity, size of follicle, and presence of leucocytes within the colloid.

RESULTS

FENCE LIZARDS (*Sceloporus occidentalis*)

The response of the thyroid in fence lizards was uniform and clearcut. A 50 per cent increase in height of the follicular epithelium was considered the minimal response, and was designated by the symbol +. A response of this degree was usually accompanied by definite changes in the colloid, such as a loss in density of stain, a tendency toward light-blueness in the Mallory stain, or a light bluish green in the haematoxylin, aniline blue, methyl green stain, and a successively more extensive vacuolization. Increases of 100, 150, and 200 per cent in follicular epithelial height and the accompanying changes in the colloid were designated ++, +++, and ++++ reactions, respectively.

No reaction whatsoever was observed in the fence lizard's thyroid after injection of as much as 56 milligrams of desiccated pituitary of sole. Amphibian pituitary (*Rana pipiens*), in contrast, produced the greatest stimulation even though the dosage given was half that of any of the other pituitary powders. In several instances after "high" dosage (28 milligrams) of pituitary of *Rana*

pipiens there was an average increase of 250 per cent in the height of thyroid follicular cells. In individual follicles, cells as high as 45μ were noted, as compared with the control height of 8μ.

Pituitary of chicken exerted only a very mild thyrotropic effect on the fence lizard's thyroid. Doses up to 56 milligrams produced only a minimal (+) reaction.

TABLE 1

ACTION OF THYROTROPIC HORMONES IN THE FENCE LIZARD (*Sceloporus occidentalis*)

Animal	Preparation	Dose*	Effect	Animal	Preparation	Dose*	Effect
C1	Ringer's solution......	2.0 cc.	0	BL4	Chicken........	5 0	0
C2	Ringer's solution......	2.0 cc.	0	BM1	Chicken	20.0	±
C3	Ringer's solution......	3.0 cc.	0	BM2	Chicken...............	20 0	+
C4	Ringer's solution......	3.0 cc.	0	BM3	Chicken	20.0	0
C5	Ringer's solution......	1.0 cc.	0	BM4	Chicken............. .	20 0	+
C6	Ringer's solution......	1.0 cc.	0	BH1	Chicken...............	56.0	+
FL1	Sole....................	5.0	0	BH2	Chicken.....	56.0	+
FL2	Sole............... ..	5.0	0	BH3	Chicken...............	56.0	+
FL3	Sole..................	5.0	0	BH4	Chicken...............	56.0	+
FL4	Sole..................	5 0	0	SL1	Sheep alc. extract.......	5.0	0
FM1	Sole..................	20.0	0	SL2	Sheep alc. extract.......	5 0	0
FM2	Sole.	20.0	0	SL3	Sheep alc. extract.......	5.0	+
FM3	Sole..................	20.0	0	SL4	Sheep alc. extract..	5 0	+
FM4	Sole..................	20.0	±	SM1	Sheep alc. extract......	20 0	++
FH1	Sole..................	56.0	0	SM2	Sheep alc. extract......	20 0	+
FH3	Sole..................	56 0	0	SM3	Sheep alc. extract......	20 0	+
FH4	Sole.................	56 0	0	SH1	Sheep alc. extract......	56.0	++
AL1	Leopard frog..........	2 5	++	SH2	Sheep alc. extract......	56.0	+++
AL2	Leopard frog..........	2 5	++	SH3	Sheep alc. extract... .	56.0	++++
AL3	Leopard frog..........	2.5	++	SH4	Sheep alc. extract... ...	56 0	++++
AL4	Leopard frog..........	2.5	+++	TH1	Schering extract.......	5 0	++++
AM1	Leopard frog..........	10.0	++++	TH2	Schering extract........	10 0	+++++
AM2	Leopard frog..........	10.0	+++	PTL1	Parkes extract	0 25	+
AM3	Leopard frog..........	10.0	++++	PTL2	Parkes extract	0 25	+
AM4	Leopard frog..........	10.0	++++	PTL3	Parkes extract	0 25	+
AH1	Leopard frog..........	28.0	++++	PTL4	Parkes extract.....	0 25	+++
AH2	Leopard frog..........	28.0	++++	PTH1	Parkes extract	5.0	+++
AH3	Leopard frog..........	28.0	++++	PTH2	Parkes extract....... ...	5 0	++++
AH4	Leopard frog..........	28.0	++++	PTH3	Parkes extract..........	5 0	++++
BL2	Chicken...............	5.0	0	PTH4	Parkes extract..... ...	5.0	++++
BL3	Chicken...............	5.0	0				

* All doses are expressed as milligrams of dry powder, unless otherwise specified.

The preparations of mammalian pituitary proved potent in the fence lizard in the dosages given. The lowest dosage of the 40 per cent alcohol powder of sheep pituitary (5 milligrams) produced the minimal response in some animals, and the highest dosage (56 milligrams) gave the maximal (++++) reaction. It must be borne in mind, however, that a 5-milligram dose of this preparation represents 150 milligrams of dried sheep pituitary, and that the average weight of the fence lizards used in this experiment was only about 10 grams. The more purified Parkes Th 9 thyrotropin proved as active as the sheep pituitary extract, since the 0.25-milligram dose (1 Parkes-Rowlands guinea pig unit) was barely stimulating, and the 5-milligram dose (20 Parkes-Rowlands units) elicited +++ and ++++ reactions in the fence lizard's thyroid. None of these reactions, however, was so intense as that produced by the smaller amount of frog pituitary.

TABLE 2

Action of Thyrotropic Hormones in the Slender Salamander (*Batrachoseps attenuatus*)

Animal	Preparation	Dose*	Effect	Animal	Preparation	Dose*	Effect
C1	Ringer's solution.......	0.3 cc.	0	BL2	Chicken.................	2.0	0
C2	Ringer's solution.......	0.3 cc.	0	BL3	Chicken.................	2.0	0
C3	Ringer's solution.......	0.3 cc.	0	BL4	Chicken.................	2.0	+
C4	Ringer's solution.......	0.3 cc.	0	BL5	Chicken.................	2.0	±
C5	Ringer's solution.......	0.3 cc.	0	BM1	Chicken.................	3.0	+
C6	Ringer's solution.......	0.3 cc.	0	BM2	Chicken.................	3.0	+
C7	Ringer's solution.......	0.3 cc.	0	BM3	Chicken.................	3.0	+
C8	Ringer's solution.......	0.3 cc.	0	BM4	Chicken.................	3.0	+
C9	Ringer's solution.......	0.3 cc.	0	BM5	Chicken.................	3.0	+
C10	Ringer's solution.......	0.3 cc.	0	BH1	Chicken.................	6.5	+
C11	(Uninjected)..	....	0	BH2	Chicken.................	6.5	++
C12	(Uninjected)......... ..	....	0	BH3	Chicken.................	6.5	+
C13	(Uninjected)...........	....	0	BH4	Chicken.................	6.5	+
C14	(Uninjected)...........	....	0	BH5	Chicken.................	6.5	++
C15	(Uninjected)...........	....	0	SL1	Sheep alc. extract.......	2.0	+
FL1	Sole...................	2.0	0	SL2	Sheep alc. extract.......	2.0	+
FL2	Sole...................	2.0	±	SL3	Sheep alc. extract.......	2.0	0
FL3	Sole...................	2.0	+	SL4	Sheep alc. extract.......	2.0	+
FL4	Sole...................	2.0	0	SL5	Sheep alc. extract.......	2.0	0
FL5	Sole...................	2.0	±	SM1	Sheep alc. extract.......	3.0	+
FL6	Sole...................	2 0	±	SM2	Sheep alc. extract......	3.0	+
FM2	Sole..................	3.0	+	SM3	Sheep alc. extract......	3.0	+
FM3	Sole..................	3.0	+	SM4	Sheep alc. extract.......	3.0	+
FM4	Sole.......	3.0	++	SM5	Sheep alc. extract.......	3.0	+
FM5	Sole..................	3.0	+	SM6	Sheep alc. extract.......	3.0	++
FH1	Sole..................	6.5	++	SH1	Sheep alc. extract.......	6.5	++
FH2	Sole..................	6.5	++	SH2	Sheep alc. extract.......	6.5	++
FH3	Sole..................	6.5	++	SH3	Sheep alc. extract......	6.5	++
FH5	Sole..................	6.5	++	SH4	Sheep alc. extract.......	6.5	+
FHH2	Sole..	6.0	++	SH5	Sheep alc. extract.......	6 5	+
FHH3	Sole....	9 0	+++	ThL1	Schering extract	0.5	++
AL1	Leopard frog....	1.0	++	ThL2	Schering extract.........	0.5	+
AL2	Leopard frog.........	1.0	++	ThL3	Schering extract.........	0 5	+
AL3	Leopard frog.........	1.0	+++	ThL4	Schering extract.........	0 5	++
AL4	Leopard frog..........	1.0	++	ThL5	Schering extract.........	0.5	+
AL5	Leopard frog...... ...	1.0	++	ThM1	Schering extract........	0.75	++
AM1	Leopard frog...........	1 5	++	ThM2	Schering extract........	0.75	++
AM2	Leopard frog..........	1.5	+++	ThM3	Schering extract........	0.75	++
AM3	Leopard frog......	1.5	+++	ThM4	Schering extract........	0.75	++
AM4	Leopard frog..........	1.5	+++	ThM5	Schering extract........	0 75	++
AM5	Leopard frog..........	1.5	+++	ThH1	Schering extract........	1.6	+
AM6	Leopard frog..........	1 5	+++	ThH2	Schering extract........	1.6	++
AH1	Leopard frog..........	3.25	++	ThH3	Schering extract........	1.6	+++
AH2	Leopard frog..........	3.25	++++	ThH4	Schering extract........	1.6	++
AH3	Leopard frog..........	3.25	+++	ThH5	Schering extract........	1.6	++
AH4	Leopard frog..........	3.25	++++	ThHH1	Schering extract........	1.5	++
AH5	Leopard frog..........	3.25	++++	ThHH2	Schering extract........	1.5	++
BL1	Chicken................	2.0	+				

* All doses are expressed as milligrams of dry powder, unless otherwise specified.

SLENDER SALAMANDER *(Batrachoseps attenuatus)*

The response of the thyroid of the slender salamander was by no means so uniform as that of the fence lizard, but it was not sufficiently variable to introduce appreciable error into the summaries for any single group of animals. Changes in the colloid, such as vacuolization and decrease in staining density, preceded changes in the follicular epithelium by a long interval, making it possible to establish reliably the minimal response at a lower level of cellular growth. The minimal (｜) response was considered to be a 30 per cent increase in follicular cell height in addition to the characteristic changes in the colloid, and in vascularity of the thyroid as a whole. Increases of 65, 105, and 140 per cent in cell height were classified as ++, +++ and ++++ reactions, respectively.

Sole pituitary, which produced no effect in the fence lizard, produced a slight but definite thyrotropic effect in *Batrachoseps*, especially in the higher dosage levels. The medium dosage (3 milligrams) and the high dosages (6.5 or 9 milligrams) yielded several mild responses (++) and two strong (+++) responses. It should be noted that this dosage is relatively large for an animal as small as *'trachoseps*, which rarely exceeds 2 grams in weight: the weight of most experimental salamanders was about 1 gram.

Amphibian pituitary had greater activity than any other pituitary preparations used in *Batrachoseps*. The smallest dosage of *R. pipiens* pituitary (1.0 milligram) produced an average increase of 75 per cent in height of the follicular epithelium of the thyroid, a ++ response; the 3.25-milligram dosage produced the only maximal (++++) reactions seen in *Batrachoseps*.

Chicken pituitary had a relatively high thyrotropic activity in the slender salamander. The 3.0-milligram dosage produced the minimal type of response, establishing the salamander and the goldfish as the most sensitive of all test animals to chicken thyrotropin in these tests.

The activity of two preparations of mammalian pituitary, (40 per cent alcohol extract of sheep pituitary, and the somewhat purified thyrotropic preparation manufactured by the Schering Corporation), was similar to that of chicken pituitary, and produced only lesser reactions in the dosages used. The Schering preparation in higher dosages (1.6 and 2.2 milligrams) was only slightly more effective than was the cruder alcoholic extract in dosage of 6.5 milligrams. The relative massiveness of this dosage of Schering mammalian thyrotropin in the slender salamander can be estimated from the fact that the effective dosage of this same preparation in 150-gram hypophysectomized rats was only 0.7 milligram, according to data kindly furnished by Dr. M. E. Simpson of the department of anatomy of the University of California.

GOLDFISH

The thyrotropic response in goldfish was very uniform and easily read. The increase of the stimulated follicular epithelium from a scarcely recognizable, very squamous type to a very high columnar type greatly facilitated the distinction and classification of the different grades of reaction. A 50 per cent increase in height of follicular cells was considered the minimal (+) response.

TABLE 3
ACTION OF THYROTROPIC HORMONES IN THE GOLDFISH

Animal	Preparation	Dose*	Effect	Animal	Preparation	Dose*	Effect
C1	(Uninjected)	....	0	RCH2	Bullfrog	20.0	++++
C2	(Uninjected)	....	0	RCH3	Bullfrog	20.0	++++
C3	(Uninjected)	....	0	RCH4	Bullfrog	20.0	++++
C4	(Uninjected)	....	0	RCH5	Bullfrog	20.0	++++
C6	Ringer's solution	1 cc.	0	RCHH1	Bullfrog	40.0	++++
C7	Ringer's solution	1 cc.	0	RCHH2	Bullfrog	40.0	++++
C8	Ringer's solution	1 cc.	0	BL1	Chicken	5.0	++
C9	Ringer's solution	1 cc.	0	BL2	Chicken	5.0	++
C10	Ringer's solution	1 cc.	0	BL3	Chicken	5.0	++
C11	Ringer's solution	1 cc.	0	BL4	Chicken	5.0	+
FL1	Fish	5.0	0	BL5	Chicken	5.0	++
FL2	Fish	5.0	0	BM1	Chicken	10.0	+++
FL3	Fish	5.0	0	BM2	Chicken	10.0	++
FL4	Fish	5.0	0	BM3	Chicken	10.0	+++
FL5	Fish	5.0	0	BM4	Chicken	10.0	+++
FM1	Fish	10 0	±	BM5	Chicken	10.0	+++
FM2	Fish	10.0	±	BH1	Chicken	20.0	+++
FM3	Fish	10.0	+	BH2	Chicken	20.0	+++
FM4	Fish	10 0	+	BH3	Chicken	20.0	+++
FM5	Fish	10.0	+	BH4	Chicken	20.0	+++
FH1	Fish	20.0	+	BH5	Chicken	20.0	+++
FH2	Fish	20 0	+	SL1	Sheep alc. extract	5 0	+++
FH3	Fish	20.0	+	SL2	Sheep alc. extract	5.0	+++
FH4	Fish	20.0	+	SL3	Sheep alc. extract	5 0	++
FH5	Fish	20.0	+	SL4	Sheep alc. extract	5.0	+++
FHH1	Fish	40.0	++	SL5	Sheep alc. extract	5 0	++
FHH2	Fish	40 0	++	SM1	Sheep alc. extract	10.0	+++
FHH3	Fish	40.0	++	SM2	Sheep alc. extract	10 0	+++
FHH4	Fish	40.0	++	SM3	Sheep alc. extract	10 0	++++
AL1	Leopard frog	2.5	0	SM4	Sheep alc. extract	10 0	+++
AL2	Leopard frog	2.5	+	SM5	Sheep alc. extract	10.0	++++
AL3	Leopard frog	2.5	±	SH1	Sheep alc. extract	20 0	+++
AL4	Leopard frog	2.5	0	SH2	Sheep alc. extract	20.0	+++
AL5	Leopard frog	2.5	+	SH3	Sheep alc. extract	20.0	+++
AM1	Leopard frog	5.0	+	SH4	Sheep alc. extract	20.0	++
AM2	Leopard frog	5.0	+	SH5	Sheep alc. extract	20 0	+++
AM3	Leopard frog	5.0	++	SHH1	Sheep alc. extract	40.0	++++
AM4	Leopard frog	5.0	++	SHH2	Sheep alc. extract	40.0	++++
AM5	Leopard frog	5.0	+	PTL1	Parkes	0 25	+++
AH1	Leopard frog	10.0	++	PTL2	Parkes	0 25	+++
AH2	Leopard frog	10.0	++	PTL3	Parkes	0.25	+++
AH3	Leopard frog	10.0	++	PTL4	Parkes	0 25	+++
AH4	Leopard frog	10.0	+++	PTL5	Parkes	0.25	+++
AH5	Leopard frog	10.0	++	PTM1	Parkes	2 5	++++
RCL1	Bullfrog	5 0	++	PTM2	Parkes	2.5	++++
RCL2	Bullfrog	5.0	++	PTM3	Parkes	2.5	++++
RCL3	Bullfrog	5 0	++	PTM4	Parkes	2.5	++++
RCL4	Bullfrog	5.0	+++	PTM5	Parkes	2.5	++++
RCL5	Bullfrog	5.0	+++	PTH1	Parkes	5.0	++++
RCM1	Bullfrog	10.0	++++	PTH2	Parkes	5.0	++++
RCM2	Bullfrog	10.0	+++	PTH3	Parkes	5.0	++++
RCM3	Bullfrog	10.0	+++	PTH4	Parkes	5.0	++++
RCM4	Bullfrog	10.0	++++	PTH5	Parkes	5.0	++++
RCH1	Bullfrog	20.0	+++				

* All doses are expressed as milligrams of dry powder, unless otherwise specified.

Increases in cell height of 200, 350, and 500 per cent, respectively, were considered mild (++), strong (+++), and maximal (++++) reactions. A major factor in the increase in cellular size appeared to be a rapid cytoplasmic accumulation of colloid-like secretion. This peculiar cytological response deserves further study in itself. Except in the more advanced grades of cellular response, the colloid did not show many changes. Vacuolization usually did not appear consistently until a 300 per cent increase in cell height had been attained. Except for a gradual decrease in density of stain, little change was noted in the colloid of Mallory-stained, stimulated thyroids. In the most active of maximally stimulated (++++) goldfish thyroids, however, a certain amount of blue could be found in addition to the predominating red color. The haematoxylin, aniline blue, methyl green stain did reveal a certain amount of "activity" in the colloid of stimulated individuals as a replacement of the purple, seen in controls, by a lightly staining blue-green type of colloid.

Sole pituitary, surprisingly, had very little effect on the goldfish thyroid. The highest dosages, 20 milligrams and 40 milligrams, elicited only a weaker (+ and ++) response.

Bullfrog pituitary (*Rana catesbeiana*) was found to be the most active thyrotropic agent in goldfish, since 5 milligrams, the smallest dosage given, uniformly produced reactions of the grades classified as ++ and +++. The dosage levels of 10 and 20 milligrams, respectively, gave maximal (++++) reactions. Leopard frog pituitary (*Rana pipiens*) was about one half as active in the goldfish as the preparation of bullfrog pituitary.

In contrast with its very low grade of activity in the fence lizard, chicken pituitary in the 10- and 20-milligram dosage range had a fairly strong thyrotropic effect in the goldfish, regularly producing a strong (+++) reaction.

The 40 per cent alcohol extract powder of sheep pituitary in dosages of 10 or 20 milligrams produced a similar grade (++) of response as the preparation of chicken pituitary. Since this dosage represents 300- or 600-milligram quantities of dried sheep pituitary, the actual thyrotropic potency of sheep pituitary in the goldfish is much less than that of a corresponding amount of chicken pituitary. The Parkes thyrotropic preparation in 2.5- or 5.0-milligram doses (10 or 20 Parkes-Rowland guinea pig units) proved almost as active as the pituitary powder of the bullfrog. Although maximal (++++) reactions have been tabulated for the Parkes preparation, just as for the bullfrog pituitary powder, this substance did not elicit so extreme an increase in height of follicular cells nor did it produce the same degree of "packing" of these cells with "secretion droplets."

GUINEA PIG

The three pituitary preparations that were available in the largest amounts were injected into the guinea pigs. Although determinations of oxygen consumption were made, they did not prove helpful, probably because of the relatively low dosages administered. The histological response of the thyroid, however, gave a uniform and reliable indicator of thyrotropic activity. A 50 per cent increase in follicular cell height, with concomitant changes in the colloid, and in general vascularity, was designated as the minimal (+) reac-

tion. Increases of 100 and 150 per cent in follicular cell height were classified as ++ and +++ reactions, respectively.

Sole pituitary, even in dosages of 300 milligrams, had no appreciable effect in guinea pigs. In one animal a slight "stimulation" was noted in the colloid but in no instance were there indications of cellular growth. Data on consumption of oxygen also indicated a lack of metabolic stimulation by fish pituitary.

Powdered chicken pituitary in the guinea pig, in dosages of 150 milligrams, was almost as active as 3 guinea pig units of mammalian pituitary extract,

TABLE 4

ACTION OF THYROTROPIC HORMONES IN THE GUINEA PIG

Animal	Preparation	Dose*	Effect on thyroid	Effect on oxygen consumption in per cent
C1	(Uninjected)	....	0	−1
C2	(Uninjected)	....	0	−1
C3	(Uninjected)	....	0	−10
C4	(Uninjected)	....	0	−9
C5	(Uninjected)	....	0	−1
C6	(Uninjected)	....	0	−3
F1	Fish	300	0	+5
F2	Fish	300	0	−9
F3	Fish	300	0	−11
F4	Fish	300	+	−9
B1	Chicken	150	+++	+2
B2	Chicken	150	++	+24
B3	Chicken	150	++	+5
B4	Chicken	150	+	+8
PT1	Parkes	0.75	+++	+8
PT2	Parkes	0.75	+++	+10
PT3	Parkes	0.75	+++	+6
PT4	Parkes	0.75	++	+10

* All doses are expressed as milligrams of dry powder.

judging from histological evidence. However, this fact was not recognizable from the data on oxygen consumption obtained from the same animals. The average histologic response to chicken pituitary was mild (++); to the Parkes Th 9 thyrotropic preparation, given in dosages of 0.75 milligrams (3 Parkes-Rowland guinea pig units), it was somewhat stronger.

DISCUSSION AND CONCLUSIONS

The data in table 5 and in the figure (p. 241) provide proof that the qualitative variations in the thyrotropic hormone of vertebrates can be revealed in physiological experiments. They show that the relative activity of thyrotropins varies extremely in different test species. For example, tested in the guinea pig, three units of Parkes mammalian thyrotropin are equivalent, approximately, to more than 150 milligrams (by calculation, about 225 milligrams) of chicken pituitary. If the activity of the thyrotropic hormones contained in these two preparations depends solely on quantity, this relation of

3 Parkes-Rowlands units of mammalian hormone to 150 milligrams of chicken pituitary should be preserved in any test animal. In the salamander, however, 3 Parkes-Rowlands units of Parkes mammalian thyrotropin are equivalent in thyrotropic activity to about 6.5 milligrams of chicken pituitary—a twenty-three to thirty-five-fold increase in the apparent activity of avian pituitary.

Another striking example is seen in comparing leopard frog and Parkes Th 9 pituitary preparations assayed first in the salamander and then in the goldfish. One salamander unit[1] of Parkes Th 9 pituitary extract is about 1.33 times larger than one salamander unit of leopard frog pituitary, but one goldfish unit of the Parkes preparation is about 60 times larger than one goldfish unit of leopard frog pituitary, a forty-five-fold change in relative activity of mammalian and frog pituitary in the two test animal species. Comparing in another way, it may be seen that one goldfish unit of leopard frog pituitary is equivalent to ten salamander units of the same substance. On the other hand, one goldfish unit of Parkes Th 9 pituitary is equal to less than one-fourth of a salamander unit (one salamander unit - 0.23 fish units) a complete reversal of the ratio of equivalence, and again a forty-four-fold change in relative activity. Study of tal 5 provides numerous other examples of even more striking changes, some as much as ninety-fold, or more, in the apparent biological activity of different thyrotropins when they are assayed in different animal species.

Because thyroids of the different test animals respond differently to the hypophyseal preparations tested, it is clear that physiologically significant qualitative differences exist between the thyrotropic factors present in the pituitaries of different classes of vertebrates.

Two interesting and unexpected results were obtained in the course of these experiments. First, as may be seen in tables 3 and 5, the goldfish appears unusually sensitive to small amounts of the thyrotropins of each of the four vertebrate classes tested. Second, of the several preparations tested, the thyrotropin of leopard frog pituitary has been shown to be the most potent of any when used in the form of dried pituitary powder.

The thyrotropic sensitivity of the goldfish seems to be a recommendation for its use in the bioassay of hypophyseal thyrotropin. In fact, this use of the goldfish has been suggested previously (Gorbman, 1940). It may be observed in table 5 that one Parkes-Rowlands guinea pig unit of mammalian thyrotropin was much more than enough to produce the minimal reaction in thyroid of the goldfish, indicating that the goldfish is a more sensitive test animal than the guinea pig to mammalian as well as to non-mammalian thyrotropins.

The unusual thyrotropic potency of leopard frog pituitary in fishes, salamanders, and lizards (table 5) agrees with the data of Adams and Allen (1942), who found this pituitary similarly active in guinea pigs. These authors report greater responses in the thyroid of the guinea pig after treatment with frog pituitary than after administration of equivalent amounts of mouse pituitary. From an analysis of the published literature, together with their own

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[1] As used in this discussion, a "unit" of a given thyrotropin when assayed in a certain animal species is the minimum quantity of that thyrotropin necessary to produce a minimal reaction in the thyroid. The definition of the minimal reaction recognized in each of the test animals is given in another part of this paper.

may be used for distinguishing the present fungus from *M. martini*
is the host upon which it is found. Of 45 specimens of *M. martini*
examined during this study, all were on leaves of species belonging
to the white oak group. (*Cf. Marsonia quercus* Pk. below.) The
38 specimens of Winter's *Marsonia quercina* studied all occurred
on leaves of species belonging to the black oak group.

Marssonia ochroleuca (Berk. & Curt. ex Pk.) Humph. ex Seym.
(*cf.* below) on *Castaneà* resembles the "black" oak fungus even
more closely than does *M. martini*, and it is surprising that speci-
mens from oak do not seem to have been given the name of the
chestnut fungus. Both *Marssonia ochroleuca* and the "black" oak
fungus differ from *M. martini* by having longer, more slender
conidia which are not constricted at the septum. The acervuli of
the chestnut fungus seem to be somewhat more shallow than those
of the "black" oak fungus, but this is not constant. Although the
chestnut fungus and the "black" oak fungus are very much alike in
every respect, with only vague differences easily accounted for by
the difference in host, the two are nevertheless here treated as sepa-
rate species. This is in keeping with the recognition of the usual
association of leaf spot fungi with narrowly restricted hosts and
also with tradition, as indicated by the failure of other workers to
identify the "black" oak fungus as *Marssonia ochroleuca*.

Part of the confusion concerning the identity of the present
fungus no doubt results from the variable nature of the acervulus,
an unfortunate phenomenon so far as taxonomy is concerned. It
may vary from a sub-sphere to a saucer in form. At maturity it is
usually opened broadly and lacks any conspicuously defined wall
of the type which would permit it to be considered a pycnidium.
Usually the fungus elements at the base of the conidiophores form
a thin basal stratum of more or less isodiametric, hyaline and in-
conspicuous cells, although occasionally there may be an increase
in density and definiteness and a slight darkening in some areas,
particularly at the base of the fructification. While the acervulus
of *M. martini* is confined to the region just beneath the epidermal
layer, the fructification of the "black" oak fungus may be confined
to this area or may form a deep cavity in the leaf tissue, hypophyl-
lous fructifications frequently forming a cavity extending to the
lower margin of the palisade layer of leaf tissue. At maturity the

fructification is usually broadly open, and the conidia are freed by splitting of the leaf epidermis over the acervulus.

An additional source of confusion concerning this fungus has been the failure of some workers to see the single septum of the conidium, a structure which frequently is difficult to see by the usual staining methods.   By the following method, used during this study, it may be observed with ease: A bit of the acervulus was obtained by use of a needle filed down to a chisel-like edge at the tip. This particle was placed on a slide, moistened with alcohol, swelled with 2 per cent KOH solution, stained with phloxine, and crushed beneath a cover slip by gentle tapping on the latter.   Measurements of conidia were then made and were found to differ very little if at all from measurements of conidia well moistened in water.   The septum could usually be discerned with some difficulty when the conidia were being measured.   Following this examination, the cover slip was removed and a drop of glycerine acidulated with acetic acid was placed on the bit of crushed acervulus.   The cover slip was replaced, and examination of the conidium then revealed that the cytoplasm had pulled away slightly from the septum, and that the latter could be seen distinctly.

During this study, it was found that specimens of the "black" oak leaf fungus had been placed in the mycological collections of this station under the following designations: *Marsonia* * *quercina* Wint., *Marssonia quercina* Wint. var. *major* Overh., *Glocosporium septorioides* Sacc., *Marssonina quercus* (Pk.) Magn., *Marssonina martini* (Sacc. & Ell.) Magn., *Septoria quercicola* Sacc., *Septoria quercus* Thuem., and *Cylindrosporium microspilum* Sacc. & Wint. Of these, the first three represent synonyms of the new combination presented below for the "black" oak fungus.   The others are misdeterminations.

To make the matter clear, the following list is presented of species which examination of specimens and/or consultation of literature has shown to be entirely distinct from the fungus occurring on leaves of trees in the black oak group: *Marssonina martini* (Sacc. & Ell.) Magn., *Marssonina quercus* (Pk.) Magn., *Marsonia matteiana* Sacc., *Phleospora hanseni* Bub., *Ascochyta quercus* Sacc. &

* *Marsonia* and *Marssonia* are earlier, now invalid, names for the present genus *Marssonina*.

work, they have constructed a list of vertebrate thyrotropins which is intended to show their relative potency. Adams and Allen state that, "such a list on the basis of all the data presented, arranged in order of descending potency of the APs would now run as follows: frog, rat, mouse, dog, pig, cattle (beef), toad (?), sheep, turkey, horse, man, rabbit, cat, pigeon, young chick (?), guinea pig, hen, and pituitaries of all of these animals except the pigeon and turkey, have been tested on guinea pigs." Since the present experiments have demonstrated the variability in potency of thyrotropins according to the assay

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TABLE 5

COMPARATIVE DOSES OF THYROTROPIC HORMONE NEEDED TO ELICIT
GRADED THYROIDAL RESPONSES*

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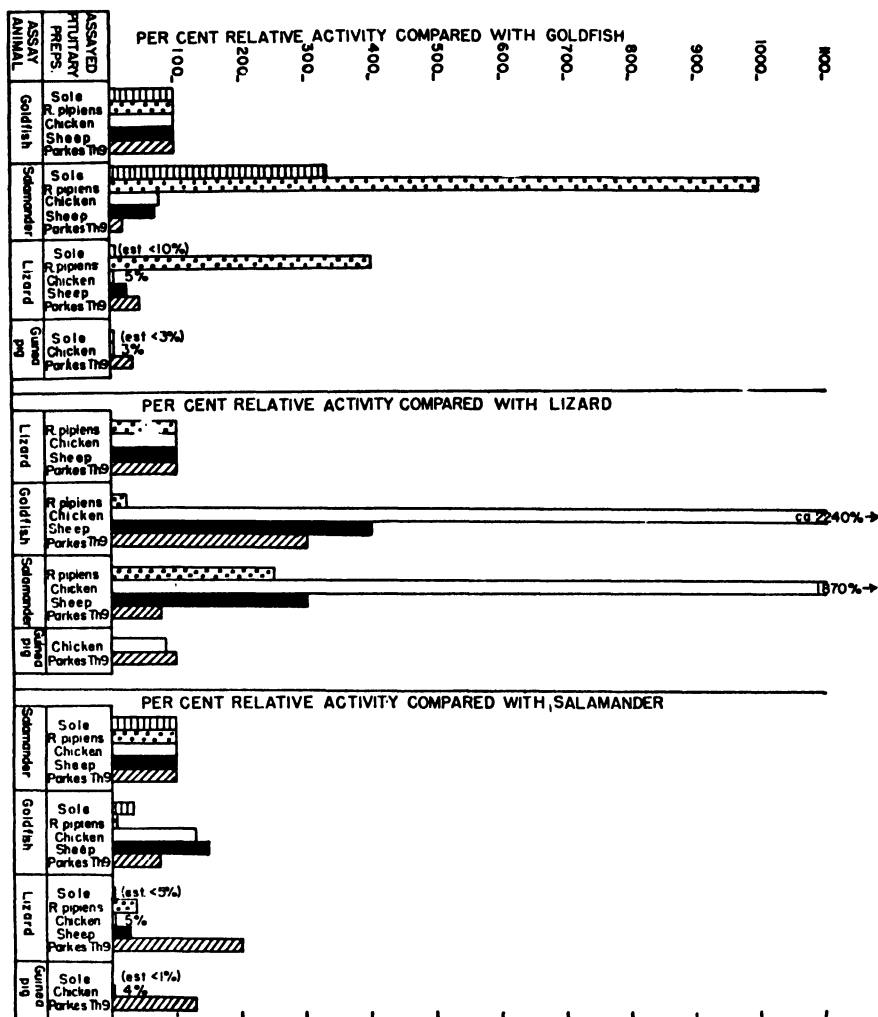| Test animal	Reaction	Sole	*Rana pipiens*	Bullfrog	Chicken	Sheep	Parkes Th 9
Guinea pig	+	a[1]		.	b		b
	++	a			150		b
	+++	a			a		0.75
	++++	a			a		a
Lizard	+	a[2]	b		56	<20 (<600)	0.25
	++	a	2.5		a	20 (600)	c
	+++	a	c		a	<56 (<1680)	<5.0
	++++	a	10.0		a	56 (1680)	5.0
Salamander	+	3.0	b		3.0	3.0 (90)	0.5
	++	6.5	1.0		>6.5	>6.5 (>195)	0.75
	+++	9.0	1.5		a	a	a
	++++	a	3.25		a	a	a
Goldfish	+	10.0	<5.0	b	b	b	b
	++	40.0	10.0	5.0	5.0	<5.0 (<150)	b
	+++	a	a	10.0	10–20	10–20	0.25
	++++	a	a	<20.0	a	40 (1200)	<2.5

* Dosage expressed as milligrams of desiccated hypophyseal tissue, except the Parkes Th 9 and sheep thyrotropins, for which the dosage is expressed as milligrams of purified powder.
Explanation of symbols used: a, highest dose given was insufficient to produce this response; a[1], highest dose given was 300 milligrams; a[2], highest dose given was 56 milligrams; b, lowest dose given was more than enough to produce this response; c, range of dosages given was too great to obtain this intermediate response.

animal used, the validity of values from other test animals in a list for the guinea pig is difficult to ascertain. In fact, it now is clear that the list prepared by Adams and Allen for the guinea pig cannot be assumed to apply to any other species. The figure (p. 241) demonstrates that if lists of relative potency are prepared for the four test animals in the present assays, entirely different orders are found in each instance.

The lack of close parallelism between the thyrotropic activities of the various assayed pituitary preparations and the taxonomic affinities of the hormonal donors and recipients seems amply illustrated by the present data. For instance, leopard frog and chicken pituitary thyrotropins proved, in the present experiments, more than twice as potent in the goldfish as sole pituitary. This situation may be contrasted with that known for the gonadotropic hor-

mones (Creaser and Gorbman, 1939). A large body of assembled data indicates for this hormone a definite tendency toward decreasing *apparent* activity when administered to species and classes further removed from the donor species. In extreme cases it is possible to administer enormous doses of gonado-

tropic hormone to species of other classes with no demonstrable gonadotropic effect (Creaser and Gorbman, 1939).

It would seem that the chemical species differences in thyrotropic hormones which are revealed by reciprocal bioassays are not great enough to cause the extreme changes in biological activity seen in the gonadotropic hormones tested in species of other classes. On this basis it is possible to explain the high thyrotropic potency of leopard frog pituitary in non-amphibian classes as the result of an unusually high content of hormone. The low potency of sole pituitary in the goldfish also may be explained, at least partly, on quantitative

grounds. For the gonadotropic factors, in which species differences appear to have a more profound effect on biological activity, even the masking effect of quantitative factors seemingly does not obscure the tendency for a given hormone to be most active in its own species and less active in unrelated species and classes.

It is of interest to consider whether protein variations in nonhypophyseal hormones can impart such variability of activity in alien species as has been shown for the thyrotropic factor. Mammalian insulin has been shown to be extraordinarily inactive in birds (Chen, Anderson, Maze, 1945) in some carefully conducted quantitative tests. The investigators entertain the possibility that this apparent inactivity of mammalian insulin in birds is due to a still undiscovered difference in carbohydrate metabolism between these species, but do not consider the possible effect of molecular differences in the hormone itself. Unfortunately, the reciprocal assay of avian insulin in mammals and birds apparently has not yet been carefully made. In a review, Haist (1944) claims that the activity of avian insulin in mammals is relatively low. Again the workers adduce, from the assay on a mammal, that avian pancreas contains very little insulin, and do not consider that avian insulin may be relatively ineffective in the mammal.

A species-variation in pituitary hormones is a factor of the highest importance in the correct interpretation of biological assays of these substances. Endocrinologists have usually assumed that a given ratio of biological units determined for a certain pituitary preparation holds true for any pituitary preparation. For example, when it is found that one guinea pig unit of sheep thyrotropic extract equals 17 chick units of the same preparation, it is common practice to presume that this same 1:17 ratio will apply to any other preparation, no matter what its source. If the hormones are qualitatively different, then this assumption is unjustified and might lead to considerable error. This point has been clearly demonstrated by the present data.

SUMMARY

Thyrotropic bioassays of seven pituitary preparations from animals representing four classes of vertebrates were made in test animals representing four vertebrate classes. Different relative potencies were found for the thyrotropins in the different kinds of test animals. It seems, therefore, that thyroids of the different test animals could be used to distinguish between qualitatively different pituitary thyrotropins from different species, reacting relatively more strongly to some than to others. If the assayed thyrotropins were qualitatively identical it would be expected that they would be also physiologically identical, and that their relative potencies would be always the same no matter what animal species was used for their assay. These data are taken as evidence of slight chemical variations of thyrotropins among species, which variations may be of significance in the interpretation of bioassay data for thyrotropic hormones.

The goldfish thyroid was shown to be an unusually sensitive test object for the assay of thyrotropic hormone. The pituitary of the frog was found to be especially high in thyrotropic activity.

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DETERMINATION AND REGULATION OF POLARITY IN THE RETINA OF HYLA REGILLA

BY

RICHARD M. EAKIN

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CONTENTS

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DETERMINATION AND REGULATION OF POLARITY IN THE RETINA OF HYLA REGILLA

BY

RICHARD M. EAKIN

STUDIES on polarity in developmental systems are numerous. Some are analyses of the physicochemical basis of polarity, as for example those of Whitaker concerning the egg of *Fucus*. The many studies of Child and his students have led to the development of the gradient theory to explain polarity and pattern in the embryo. Still other investigations, such as those of Spemann and Harrison and their followers, have analyzed the interrelationships of the parts of a developing system and the nature of regulatory processes with respect to the establishment of polarity and specific organic form. The study presented below belongs to the latter type. It is an attempt to ascertain the time and conditions in the development of the amphibian retina when its polarity becomes determined, that is, will no longer regulate if its inner and outer surfaces are reversed.

MATERIAL AND METHODS

Embryos and larvae of the Pacific tree frog, *Hyla regilla,* were used in all experiments. The age of the material for any experiment described below will be given according to a series of stages in the normal development of *H. regilla* (see pls. 22–24), numbered to correspond with the stages given by Pollister and Moore (1937) for *Rana palustris* and by Shumway (1940, 1942) for *R. pipiens.* The series of stages for *R. palustris* and *R. pipiens,* as well as Harrison's stages for *Amblystoma maculatum (punctatum),* are reproduced in Hamburger's manual of experimental embryology (Hamburger, 1942).

In most of the experiments one type of operation was performed, namely, the rotation of the presumptive or differentiated retina so as to reverse the positions of inner (vitreal) and outer (tapetal) surfaces. Operations on stages 16, 17, and 18 (pl. 22, figs. 3–8), stages in which the optic vesicle develops, were performed as follows. The ectoderm in the region of the right eye was removed; the distal half of the right optic vesicle (presumptive retina) was cut out, and was replaced after reversal of its inner and outer surfaces. The inverted retina was held in place by a glass bridge. In some operations the anteroposterior axis also was reversed, in others the dorsoventral axis. Operations on stages 19, 20, and 21 (pl. 23, figs. 9–14), stages in which the optic cup develops, required slight modification in method. Before rotation of the presumptive retina, the overlying ectoderm, lens vesicle, and rim of the optic cup were carefully removed. The thick retinal disk was then lifted from the tapetal cup and replaced, its inner and outer surfaces reversed. In order to prevent loss of the retinal disk in stages 20 and 21, it was necessary to cover the operated eye with a piece of ventral ectoderm, taken usually from the belly of an embryo in stage 18 or 19. In the earlier series (stages 16–19) ectoderm regenerating from the margins of the wound quickly covered the inverted retina.

It was difficult to perform the same operations on stages 22, 23, and 24 (pl. 24, figs. 15–20), owing to the behavior of the material at these ages. For example, as soon as the retina in these larvae is lifted from the tapetal cup the latter begins to contract and the former to spread into a large thin disk. Moreover, ectoderm from the belly of another embryo, intended as a covering for the operated eye, begins to curl as soon as it is removed. It was impossible to reverse the extirpated retina, place it in the tapetal cup, cover the site with ectoderm, and apply a glass bridge before these changes took place. Consequently, the operation on these stages was performed in steps. One larva, the host, was anesthetized with etherized water. The skin, cornea, lens, and iris of its right eye were trimmed away as before and the retina was carefully removed without injury to the tapetum. A small glass bead of the proper size was inserted into the tapetal cup and held in place by a glass bridge. The bead prevented, in large measure, the shrinkage of the tapetal cup. From another

Figure A

anesthetized larva, the donor, an entire eye was removed and gently denuded of its tapetum. A large piece of ectoderm from the belly of a third embryo in stage 18 or 19 was removed, trimmed, and placed near the host for subsequent use. The eye from the donor was then transected so as to divide the eye into a distal part including iris and lens and a proximal part containing most of the retina. The latter was quickly inverted into the tapetal cup of the host in place of the glass bead, covered with the piece of ventral ectoderm, and held in place by pressure of a glass bridge.

In all the operations just described, a pair of microscissors (fig. A) was useful. The scissors were made from two small pieces of razor blade soldered to the tips of a pair of forceps in the manner described by Burch (1942) for making microscalpels. The cutting edges of the pieces of razor blade were directed inward and the tips of the blades were ground to very fine points on a powered carborundum wheel, the speed of which could be controlled by a foot treadle. The blades were brought into a shearing plane by bending carefully one or both of the arms of the forceps. The instrument, together with a hair loop, was used in several ways: as scissors for shearing a layer of cells; as forceps for picking up, clearing away, or manipulating cells; and—one blade only of the microscissors—as a microscalpel for cutting or piercing. The instrument was easily made and could be quickly resharpened if the tips became blunt or broken. It should be a useful tool in other investigations in experimental embryology.

Experimental animals were kept for one postoperative day in finger bowls filled with sterilized Holtfreter's solution diluted by half with water. They were then transferred to pond water on the second day and usually were fixed in Bouin's solution on the tenth postoperative day. Fixed material was sectioned and was stained with Harris' haematoxylin and eosin. A total of 218 successful operations were performed, the majority of them involving a reversal of the retina; 179 experimental animals were sectioned and examined histologically.

RESULTS AND CONCLUSIONS

The experimental animals were classified according to the following types of structural organization in the inverted retina: good regulation, fair regulation, reversed polarity, and disorganization.

TABLE 1

RESULTS OF REVERSING THE PRESUMPTIVE OR DIFFERENTIATED RETINA
IN EMBRYOS AND LARVAE OF HYLA REGILLA

Stage (series)	Number of specimens	Good regulation	Fair regulation	Reversed polarity	Disorganization
16	39	14	9	0	16
17	21	17	4	0	0
18	24	9	10	0	5
19	16	5	5	3?	3
20	21	4	7	2?	8*
21	12	0	2	2	8*
22	8	0	1?	7	1

* Includes specimens exhibiting vesicle formation (see p. 251)

1) *Good regulation* meant that the operated eye appeared normal with respect to the general morphology of the eye and the histological differentiation of the retina. Tapetum, iris, and pupil were well formed and in typical relation to the normal pattern of rods and cones, bipolar neurones, and ganglion cells. Examples of good regulation may be seen in figures 21 to 25 (pl. 25).

2) *Fair regulation* included instances of poor ocular morphology either in the pigmented parts of the eye or, more commonly, in the form and position of the retina but with the polarity of the retina normal, that is, with rods and cones proximally situated and next to the tapetum, and the ganglion cells distally situated and bordering the vitreous body. An example of fair regulation is shown in figure 27 (pl. 26). In both this and the preceding type of organization, presumptive sensory sells differentiated neighborwise (Needham, 1942) into neurones, whereas presumptive ganglion cells became rods and cones. This type of regulation has been designated "autonomous histological or material regulation" by Holtfreter (1938) and "qualitative regulation" by Eakin (1939), in contrast to adjustments in the size and shape of an embryonic part, termed "autonomous morphological regulation" by Holtfreter or "quantitative regulation" by Eakin.

3) *Reversed polarity* implied an absence of regulation in the inverted retina because sensory elements were situated distally and the neural elements proxi-

mally. Figure 31 (pl. 27) illustrates an example of reversed polarity. Unlike the first two classes given above, the retinal cells in this type differentiated in accordance with their prospective significance, that is, selfwise (Needham, 1942), or according to their position in the developing eye before the operation.

4) *Disorganization* included specimens with an experimental eye so disarranged, particularly the retina, that it was impossible to state whether the sensory and neural elements showed a normal or an inverted pattern. An instance of disorganization is illustrated in figure 30 (pl. 26).

Table 1 presents a summary of the results of reversing the presumptive retina in embryos and larvae of *Hyla regilla* varying in age from stage 16 to stage 22 (see pls. 22–24).

Experiments on Nonmotile Embryos

Retinae inverted in the nonmotile stages (16 and 17) showed normal polarity in 73 per cent of the specimens (see table 1) and disorganization in 27 per cent; there was no instance of reversed polarity. Of the examples exhibiting histological regulation, 70 per cent showed well-formed eyes. Stage 16 appeared to be a less favorable time for performing the operation than stage 17, owing probably to the fact that, at the earlier stage, brain was frequently and inadvertently included in the piece of early optic vesicle which was inverted. In these animals dislocated pieces of neural tissue were commonly found adjoining or near the experimental eye. The operation could be performed with greater precision in stage 17, in which the optic vesicle was larger, better defined, and more accessible. Apparently only presumptive retina was reversed in animals of this series, because nervous tissue was not found connected to or in the near vicinity of the operated eye. It may be concluded that the presumptive retina in the optic vesicle of the nonmotile embryo of *Hyla regilla* is highly regulatory.

Examples of good regulation in experiments on the nonmotile embryo are shown in figures 21 and 22 (pl. 25), representing respectively operations performed on stages 16 and 17. It will be noted that ocular morphology is excellent and, aside from slight irregularities in the retina such as that shown in the ganglion layer in figure 22, the histological picture is normal. Optic nerves were present, although they are not shown in these sections. The experimental eyes were almost indistinguishable from the control eyes. They differed from the norm in being slightly smaller in size and in having a smaller lens. Figure 23 (pl. 25) shows an eye, operated upon in stage 16, in which good regulation has taken place despite the accidental inclusion of parts of the brain with the rotated piece of optic vesicle. Dorsal and ventral to the eye lie the ectopic masses of nervous tissue. Note the patent optic stalk.

Experiments on Early Motile Embryos

As a result of operations performed on the flexure and coil stages (18 and 19), 73 per cent of the retinas exhibited normal polarity (see table 1) and 27 per cent showed disorganization, including three doubtful instances of reversed polarity, to be considered later (p. 254). The operated eye in 48 per cent of

the animals showing histological regulation of the inverted retina possessed excellent to fair morphology. From these data it appears that the polarity of the retina is still undetermined at stage 19, in which the optic cup is differentiated into an outer thin tapetum and an inner thick sensory layer.

Examples of good regulation in this series may be seen in figures 24 and 25 (pl. 25), illustrating respectively operations performed on stages 18 and 19. General morphology in both is good and the histological pattern in the retina is excellent (fig. 25) or good (fig. 24). In the retina of the latter example the outer molecular layer is not well differentiated and there appears to be a vesicle lined with rods and cones situated within the outer nuclear layer. Upon tracing this vesicle, it was found to be a pocket or recess formed by an infolding of the receptor layer. Figure 25 shows some of the finer details of the rods and cones, namely, the clear ellipsoid, the thick outer segments of the rods, and the narrow and tapering outer segments of the cones. Optic nerves were present in both specimens although they are not shown here.

EXPERIMENTS ON EARLY SWIMMING LARVAE

Of 21 larvae in which the retina was inverted in stage 20, 11 (or 52 per cent) showed normal polarity, 2 (or 10 per cent)—but it should be mentioned that one of these is doubtful—showed reverse polarity, and 8 (or 38 per cent)—including 7 in which an epithelial vesicle formed, a condition to be described later (see p. 251)—showed disorganization. Only 36 per cent of the experimental eyes showing histological regulation possessed good morphology.

Of larvae in which the retina was reversed at stage 21, only 2 (or 17 per cent) showed fair regulation and none exhibited good regulation; 2 (or 17 per cent) showed reversed polarity; disorganization of the retina and, incidentally, vesicle formation, were exhibited by 8 (or 66 per cent) of the 12 specimens available for study.

Although the number of interpretable examples in the two series given above was limited, owing to a high incidence of disorganization and vesicle formation, the results indicate, nevertheless, that in stage 20 the retina is still capable of regulatory development. Figure 26 (pl. 26) is an example of good regulation, one of four from series 20. The general morphology of the eye is fair, although not so good as that in examples from other series. The retina is essentially normal in its histological picture, although irregularities are present, as, for example, the displacement of the central part of the inner molecular layer, the isolated patches of white matter, and the absence of sensory elements in the central section of the retina. An equally good, if not better, picture of regulation was provided by another specimen (unfigured), which, like the one shown in figure 26, possessed a lens. The other two animals from series 20 classified as showing good regulation lacked lenses and their experimental eyes were not so well formed. This feature and others to be presented later point to the lens as an important morphogenic factor in the development of the eye in *Hyla*.

The fact that no examples of good regulation and only two of fair regulation appeared in the group of larvae in which the retina was reversed in stage 21

suggests that at this stage of development the polarity in the retina of *Hyla regilla* is becoming irreversibly determined. Moreover, in two specimens there was evidence of a reversed pattern of sensory and neural elements. It may be recalled that at stage 21 in the normal development of *Hyla* there appears the first visible indication of retinal differentiation (see pl. 23, fig. 13), namely, the outgrowth of axons from the ganglion cells to form the optic nerve. In all probability other nerve fibers are forming at this time, although they could not be observed in my preparations. Stage 20 thus appears to be the last period in the development of the eye of *Hyla regilla* which permits a histological (qualitative) regulation along the polar axis of the retina.

EXPERIMENTS ON OLDER LARVAE

In eight successful operations on larvae in a later swimming stage (22) all the specimens except one exhibited reversed polarity (88 per cent); in the disorganized exception normal polarity existed in certain regions only of the retina. The data are limited, owing to the fact that the operation was difficult to perform (see p. 246), but it is fairly clear that by stage 22, in which the retina begins to differentiate into layers, the polarity of the retina is irreversibly determined and regulatory development is no longer possible under the conditions of this experiment.

Examples of reversed polarity are shown in figures 28, 31, and 32 (pls. 26 and 27). In the first (fig. 28, pl. 26), one of the best in the series, it will be noted that the rods and cones are situated distally whereas the layer of ganglion cells is proximal in position. Sensory elements, outer and inner molecular layers, and outer and inner nuclear layers are well differentiated; aside from an inverted seriation they are in typical order. The ganglion layer, however, is abnormally thick. The optic nerve arises in the ganglion layer, passes *distad* through the retina, penetrates the tapetum, and emerges beside an enlarged blood vessel or sinus situated below the skin. The optic nerve may be traced for many sections; it bends posteriorly, runs along the outer surface of the tapetum, and finally ends abruptly at the caudal limit of the eye. The tapetum, entirely of host origin, is a thick pigmented layer which completely envelops the retina. Although the outer segments of rods and cones are shown only below the optic nerve in the section figured, other sections show many well-differentiated receptor cells above the nerve.

In figure 31 (pl. 27) may be seen another experimental eye exhibiting reversed polarity, that is, one in which the inverted retina has not regulated. The retina is well differentiated into the typical sensory and neural layers. Moreover, this particular section shows good ocular morphology despite the presence of an incomplete iris and an abnormally large space between the retina and the tapetum. In this specimen, unlike the one shown in figure 28, the tapetum surrounding the retina is of donor origin, having been transplanted along with the graft. The tapetum of the host, instead of retaining its cuplike shape, has formed several rounded masses of pigmented cells at the back of the orbit. Some of these may be observed proximal to the eye. An optic nerve is not present.

Figure 32 (pl. 27) illustrates how a donor tapetum, transplanted with the inverted retina, has fused with the tapetal cup of the host to form an unbroken sphere of pigmented epithelium. Within this vesicle the retina has differentiated in accordance with its original polarity, that is, without any histological (qualitative) regulation. Receptor cells, although not fully formed, are easily recognizable. They lie on the distal surface of the retinal mass, their stunted outersegments projecting into the cavity between the retina and the tapetum. In the central area the layer of sensory cells is deeply folded or invaginated. In places, a narrow outer molecular layer proximal to the nuclei of the rods and cones may be seen. Inner nuclear, inner molecular, and ganglion layers may be identified in spite of marked irregularities. Important features are the unbroken serial order of the layers and the unmistakably reversed polarity of the retina. An optic nerve is not present.

The general conclusion, based on the evidence presented above, is that, under the experimental procedure of rotating the developing retina so as to reverse the inner and outer surfaces, the polarity of the retina in *Hyla regilla* becomes determined at stage 21. Prior to stage 21 the retina exhibits both morphological and histological regulation; thereafter, regulation along the polar exis does not occur. There remain to be presented, however, additional observations and a consideration of exceptional cases, before this conclusion will be discussed and related to other studies.

VESICLE FORMATION

It was noted earlier (see page 249 and the footnote to table 1) that many of the specimens in which the retina was reversed in stages 20 and 21, although classified as exhibiting disorganization, actually presented a different picture. In these specimens, a structure best described as an epithelial vesicle or cyst developed from the inner cells of the embryonic skin which had been grafted over the inverted retina or from migrating mesodermal cells of the host. Among the 8 animals listed as showing disorganization in series 20, 7 exhibited this picture of vesicle formation. The cyst apparently formed first as a fluid-filled chamber separating the retina from the overlying skin. Into this thin-walled, eventually pigmented, vesicle the retina developed as a spherical or ellipsoidal body surrounded on all sides by fluid except on its proximal surface, where it was in contact with the tapetum, and this latter, instead of retaining its originally cup-shaped form, became condensed to several rounded masses of pigmented cells. These features are shown in figure 34 (pl. 27), a photomicrograph of one of the 7 specimens referred to above. One may observe further that, in section, the retina has the appearance of a horseshoe the ends of which are situated proximally and in contact with the tapetal mass. Typical differentiation of sensory and neural layers has occurred, although the rods and cones are not well formed. The position of the layers is of primary importance. The layer of rods and cones is outermost; the ganglion cells, which line the cavity of the horseshoe-shaped retina, are innermost. It would appear, therefore, that the inverted retina in the above-mentioned example, and in the 6 similar ones, had differentiated in accord-

ance with its original polarity and that no histological regulation had occurred. This conclusion is the opposite of that based upon findings in 11 specimens of the same age group (see table 1 and p. 249), in which the epithelial cyst did not appear. This discrepancy was disturbing and was not understood until certain control experiments, now to be described, had been performed.

CONTROL OPERATIONS

Twenty-two control operations were performed in which young larvae in stage 20 were prepared for retinal inversion as described earlier. The retina, however, was not reversed as in the experimental series, but was left undisturbed and was covered with embryonic skin from the venter of an embryo in stage 18 or with ventral ectoderm from young larvae (stage 21). In 12 of the 22 specimens an epithelial vesicle did not form and the retina differentiated normally with respect to the position of its layers. Most of the eyes, although small and lacking lenses, were of good form. In the other 10 controls, however, a cyst developed and the retina was essentially like that in the 7 experimental animals of series 20 which possessed an epithelial vesicle.

The striking similarities between control and experimental animals in which the epithelial vesicle was present may be seen by comparing figure 35 (pl. 27), an example of the group of 10 controls, with figure 34 (pl. 27), an example of the 7 experimental animals. The nature of the cyst, the positions of retina and tapetum, and the arrangement and form of sensory and neural layers are almost the same. In both, the fibers of the optic nerve pass mesiad from the ganglion layer, leave the retina near the ends of the "horseshoe," penetrate the condensed mass of tapetal cells, and continue on to the brain. The optic nerve is always smaller in size, however, than that of the normal eye. It would appear, therefore, that in the controls possessing an epithelial vesicle the retina had differentiated into a reversed pattern of sensory and neural elements.

This interpretation is probably not correct. It seems more likely that in the controls, in which the fluid-filled vesicle developed, the retina became folded into the form of an inverted U. There are several observations which support this view. A few specimens fixed several hours or a day after the control operation showed the early presence of a space between the retina and the engrafted embryonic skin and a tendency for the retina to curl distad, particularly along its dorsal margin, into the developing visicle. The ventral margin seemed to be well anchored by the developing optic nerve. A mesiad curling or ventrad recession of the dorsal part of the tapetum permitted the retina to change its form and position. The ventral half of the tapetum, however, did not usually become dissociated from the retina. These features are shown in figure 38 (pl. 28), a photomicrograph of a control specimen fixed twenty-four hours after the operation. It is assumed that this curling of the retina would have proceeded until the retina was folded upon itself. As a result, prospective sensory cells, once proximal in position, would have been carried laterally and ventrally and would have become the outer sensory surface of the retinal "horseshoe." The tendency of an explanted retina to spread and curl was noted

in operations performed on larvae in stages 20 and 21 and was found to be marked in stage 22.

A number of specimens from the group of 10 controls fixed at the usual time, ten days after operation, gave unmistakable evidence that curling had occurred as described above. In one specimen, shown in figure 39 (pl. 28), the retina has the appearance of a question mark. It had curled distad at the dorsal margin of the optic cup. If the epithelial vesicle had been larger, the retina would probably have folded into the horseshoe-shaped form which it exhibited in most of the 10 controls. Curling of the ventral margin of the retina was apparently prevented by the anchoring effect of the optic nerve (not shown) and by the maintenance of the cuplike form of the ventral tapetum. The small patch of pigmented cells on the dorsal rim of the iris was probably torn away from the tapetum when the retina began to curl outwardly. Figures 38, 39, and 34 may be regarded as steps in the development of the cyst and the inverted U-shaped retina.

It seems, therefore, that the formation of a fluid-filled cyst distal to the retina, whether the latter is reversed or undisturbed, presents a different situation from that found in experimental or control animals in which the cyst does not develop. In the absence of a cyst the polarity and arrangement of sensory and neural elements of the inverted retina of stage 20 become regulated into the structural pattern characteristic of the normal eye. The unreversed retina of the control, however, develops normally in the absence of the epithelial vesicle. On the other hand, the presence of a cyst does not provide the proper conditions for regulation within the inverted retina, and that which appears to be reversed polarity in the controls in which the cyst appears is actually normal differentiation of retinas which become inverted through curling. It will be suggested later (p. 259) that a close relationship of the inverted retina to the tapetum is essential for regulation and that the absence of regulation in experimental animals which develop a cyst is owing to an absence of this relationship.

There seemed to be little or no relation between the presence of a cyst and the presence or absence of mesoderm included with the ventral ectoderm that was used to cover the wound. A cyst developed in several specimens in which the ventral ectoderm had been carefully cleaned of adhering mesodermal cells. The vesicle formed also in specimens in which many mesodermal cells were transplanted with the ventral ectoderm. Unfortunately, it was not always noted in the protocol whether the graft used to cover the wound of an experimental or control animal included mesoderm. The data which are available, however, are given in table 2. It will be noted that, of 12 specimens in which the ectoderm was free of mesoderm, 4 exhibited cysts and 8 did not; of 9 in which mesoderm was included with the ectoderm, 7 formed cysts and 2 did not. Although the data were analyzed statistically, it was thought that because the samples were small and because certain subjective factors could not be shown by numbers, my judgment was more reliable than some index of correlation.

Some evidence is available on the possible relation between vesicle forma-

tion and the age of the ventral ectoderm used to cover the wound. In some experimental and control operations ventral ectoderm from stage 18 was used to cover the wound; in others ventral ectoderm from stage 20 was used. In the last group donor and host were of the same age and usually from the same clutch of eggs. Cysts appeared in several examples of both groups. Apparently the age of the ectoderm was not a factor in the development of the cyst.

Whether or not an epithelial vesicle forms is probably owing to the degree of trauma to blood vessels supplying the orbit. If cut or torn vessels remain open after the operation, hemorrhage under the engrafted skin leads to the formation of the cyst. That the fluid in the cyst is for the most part blood is supported by several observations. First, it was noted that sometimes the cyst appeared within a few hours after the operation; if the young vesicle was punctured, fluid and corpuscles escaped. Secondly, some experimental and

TABLE 2

VESICLE FORMATION AND THE PRESENCE OR ABSENCE OF MESODERM

(Experimental and control animals, stage 20)

	Total	Vesicle present	Vesicle absent
Ectoderm without mesoderm.............	12	4	8
Ectoderm with mesoderm................	9	7	2

control animals, a few of which were fixed within the first twenty-four hours after the operation, showed the presence of blood corpuscles in the cyst. For example, in figure 38 (pl. 28), a photomicrograph of a control fixed a day after the operation, may be seen a large space between the retina and engrafted skin which contains a network of coagulated material and several masses of blood corpuscles (*bc.*). The single row of cells beneath the ectoderm and those adjacent to the ganglion cells of the retina are mesodermal cells (*m.*) which had been transplanted with the ectoderm. Another group of mesodermal cells and blood corpuscles may be seen on the dorsal aspect of the eye. The initial curling of the uninverted retina and the recession of the dorsal part of the tapetum have already been discussed.

INSTANCES OF UNCERTAIN CLASSIFICATION

Returning to the summary of the results given in table 1, it will be noted that of the 121 experimental animals in which the operation was performed before stage 21 only 5 showed any evidence of reversed polarity. Of these, three from series 19 were listed as doubtful cases of reversed polarity. Several sections from one of these showed an inverted pattern of sensory and neural cells. On the whole, however, the eye exhibited a highly irregular and unorganized internal structure and could have been listed among those showing disorganization. This specimen is not regarded as significant. The other two doubtful specimens from series 19 were specimens in which the experimental procedure had been modified. The ectoderm from the right side of the head had been removed as usual, but the lens vesicle had not been extirpated. Instead, it had

been rotated together with the retina to a position next to the tapetum and proximal to the retina. Here, buried within the eye, the vesicle developed into a crystalline lens and exerted an influence upon the overlying retina, which, instead of regulating, continued its course of differentiation in accordance with its original polarity and its normal relationship to the lens. Factors favoring a histological regulation of the retina in relation to the cornea and iris were also operative. This is especially well shown in figure 29 (pl. 26), a photomicrograph of an experimental eye in which the retina *with lens vesicle* had been inverted at stage 19. Note the position of the lens on the inner, proximal side of the eye. Although it does not appear to be fully formed in the section figured here, other sections show that lens fibers are well differentiated. The inner half of the retina is oriented with respect to the lens; the outer half of the retina, however, is oriented in relation to the primary iris, pupil, and cornea. Each half has a normal pattern of rods and cones, nuclear and molecular layers, and ganglion cells. They differ, however, in the polarity or order of arrangement of the cells. For example, the receptors, which lie back to back, are distally situated in the inner half of the retina (reversed polarity) and proximally situated in the outer half of the retina (histological regulation). This specimen and the one like it show results that are in agreement with those obtained in other experimental animals of this series so far as the outer retina is concerned, and the reversed polarity of the inner retina is owing to the orienting influence of the lens which had been rotated with the retina.

Certain other features of the figure just discussed may be mentioned. A secondary pupil, iris, and cornea (?) have formed in relation to the abnormally placed lens. The organization of the tapetum, as well as that in the retina, has thus been modified by the presence of the lens. No lens is present at the usual site—a feature observed in many specimens, both experimental and control, in which the lens anlage or vesicle had been removed (see p. 256). The optic nerve arises from the ganglion layer of both inner and outer retinal halves at a posterior level of the eye, where the retina is much more irregular and not so clearly organized into two halves.

The last two of the five exceptional examples mentioned above are instances of reversed polarity in series 20. In one of these a lens appeared deep in the orbit and proximal to the retina. Although according to the protocol an attempt had been made to remove all lens cells before the retina was reversed, apparently some cells were unintentially translocated with the retina. As in the two instances of intentional transplation of the lens in series 19, here also the inner half of the retina became organized about the lens and exhibited an inverted polarity of the retinal elements. The specimen was similar to that shown in figure 29 (pl. 26). The other instance of inverted polarity is less easily explained. It will be noted that in this specimen (fig. 40, pl. 28) the rods and cones lie at the distal surface of the retina whereas the ganglion cells are proximal in position. A large optic nerve goes to the brain by an unusual route: it does not pass through the retina and tapetum, but instead runs from the mesially situated ganglion layer directly to the optic chiasma. Other sections

of the eye show the reversed polarity of the retina better than the one figured; still other sections, however, exhibit considerably more chaotic organization in the retina than that seen here. Although the protocol seems clear on the point of age of the larva at the time of the operation, it is possible that this specimen was somewhat older than the others in the series. In this event, the polarity of the retina may have been fixed before the retina was inverted. This interpretation, however, provides no explanation for the course of the optic nerve, which, unlike that exhibited by another example of reversed polarity (see fig. 28, pl. 26), does not pass distad through the retina, but instead runs mesiad directly to the brain.

It has already been pointed out that the one doubtful case of fair regulation listed in table 1 under stage 22 is an example of disorganization in which a few of the sections showed a normal polarity in the structure of the inverted retina. This specimen is regarded as not significant.

PRESENCE OR ABSENCE OF LENS

Data on the presence or absence of a lens in experimental and control animals are given in table 3. It will be noted that in every series, with the exception of 21 and 22, a lens is present in one or more specimens, although the percentage of animals showing a lens decreases steadily from series 17 to series 21, that is, from 95 per cent of the specimens upon which the operation was performed in stage 17 to 0 per cent of the specimens upon which the operation was performed in stages 21 and 22. The 22 controls (see p. 252), in which the retina was not inverted but in which the lens vesicle was removed at stage 20, are also included. Series 16 showed a much lower incidence (46 per cent) of lens formation than series 17, owing probably to the difficulty of performing the operation with precision in stage 16. Parts of the brain were often included in the rotated piece of optic vesicle; sometimes the inverted presumptive retina became incorporated into the wall of the brain, or was even included within the ventricles of the brain (see fig. 33, pl. 27).

Possible sources of the lens, when present, include the following: (1) Wolffian regeneration, (2) regeneration from fragments of the original lens anlage or vesicle, and (3) induction of a lens in the ectoderm covering the inverted retina. It has been shown that Wolffian regeneration does not occur in certain anurans (Stone and Sapir, 1940), and I have seen no evidence which would lead me to believe that *Hyla regilla* differs from other anurans in this regard. With few exceptions the operative procedure called for the complete removal of the lens anlage or vesicle. In a few examples, such as the one shown in figure 29 (pl. 26), especial care was taken not to injure the lens vesicle which was rotated with the retina. In most other specimens, however, it is doubtful that fragments of the lens rudiment remained after the operations had been completed. This leaves the third alternative as a probable explanation for the presence of the lens in a large number of experimental eyes.

The decline in incidence of lens formation with increasing age of the experimental series may be attributed either to diminishing competence in the ectoderm to form lens or to a decrease in the inducing power of the retina. That

it is probably the latter is suggested by the fact that lens failure occurred in experimental animals of series 21 and 22 although the inverted retina was covered with young ventral ectoderm (stage 18). This reasoning, however, is not altogether valid unless ventral ectoderm of *Hyla regilla* is known to be competent for lens formation. Ventral ectoderm might be refractory to lens induction, as it is in *Rana esculenta* (Spemann, 1912a). Moreover, mesoderm was frequently transplanted with the ectodermal graft used to cover the wound, and it might have acted as a barrier to the inductive influence of the retina upon the ectoderm. It may be significant that mesoderm was absent from the graft in the eight specimens in which the lens occurred, in the older experimental and control animals (last four series of table 3). It is also sig-

TABLE 3

PRESENCE OR ABSENCE OF LENS IN EXPERIMENTAL AND CONTROL ANIMALS

Stage (series)	Number of specimens	Lens present	Percentage of lens present	Lens absent
16.	39	18	46	21
17.	21	20	95	1
18.	24	18	75	6
19.	16	11	69	5
20.	21	4	19	17
20 (control).	22	4	18	18
21.	12	0	0	12
22.	8	0	0	8

nificant that in no specimen, experimental or control, in which the epithelial cyst formed, did a lens appear. Apparently the accumulation of fluid (blood) between retina and ectoderm prevented the induction of a lens. In some, however, lens formation failed in both experimental and control animals despite the absence of both cyst and a layer of mesoderm separating retina and ectoderm. Data are limited, unfortunately, because the problem of lens induction was incidental to the study of polarity in the retina.

OPTIC NERVE

An optic nerve was present in the majority of the experimental eyes. For example, every specimen in series 17, 18, and 19 had a large optic nerve running to the right side of the brain. In many specimens the nerve was as large and as well formed as the left nerve from the control eye; in some, however, the nerve from the experimental eye was smaller. The failure of the nerve to form in about one-third of the experimental eyes of series 16 is undoubtedly owing to the high incidence of chaotic organization in this series (see table 1, p. 247) and to development of the eye within the walls or ventricles of the brain (see pl. 27, fig. 33). An optic nerve was present in all but three specimens of series 20, and likewise in all but three in series 21. Those eyes in which the nerve failed to form, in these series, were of very small size and were disorganized. Among the experimental eyes of series 22, however, only one (see pl. 26, fig. 28) exhibited an optic nerve.

It is a known fact that the ganglion layer of the retina degenerates in eyes lacking a functional optic nerve. This feature was observed in a number of experimental eyes studied. For example, the eye seen in figure 23 (pl. 25), one of several specimens from series 16 lacking an optic nerve, shows degenerating ganglion cells (*g.*). Many nuclei are pycnotic or fragmented; some have probably disappeared already. The eye contained within the ventricle of the brain (pl. 27, fig. 33) has no optic nerve, as noted above, and the ganglion layer is represented by only a sparse line of nuclei lining the retinal vesicle. By contrast, figures 34 and 35 (pl. 27) show thicker ganglion layers in eyes possessing optic nerves but developing in a situation somewhat similar to that shown in figure 33. Incidentally, the scattered spaces in the inner nuclear layer of all three are probably owing to the separation of cells as a result of the retina's developing in a large fluid-filled cavity. In figure 36 (pl. 28) may be seen another instance of degeneration of ganglion cells. The eyecup to the left in the photomicrograph does not possess an optic nerve. Note the absence of nuclei in the central part of the ganglion layer and the large number of pycnotic and fragmenting nuclei above and below the letter *g.* An optic nerve and a very thick ganglion layer are present in the eye to the right. It should be noted, however, that not all eyes lacking an optic nerve exhibited degeneration of the ganglion layer. For example, the eye shown in figure 31 (pl. 27) had not a vestige of an optic nerve and yet the ganglion layer is thick and apparently in good condition.

The course of the optic nerve in some of the experimental eyes was noteworthy. Usually the path of the nerve from ganglion layer to optic chiasma was normal. Connection with the brain was not made except by way of the optic chiasma, but not infrequently the exit of the nerve from the eye was unusual. Mention has already been made (p. 256) of an eye, exhibiting reversed polarity of the retina, in which the optic nerve (*n.*, pl. 28, fig. 40) passed directly to the brain from the medially situated ganglion layer. Normally (see fig. 24, pl. 25) the nerve passes through the various layers of the retina and tapetum en route to the brain. The two instances of reversed polarity in series 21 exhibited a similar direct passage of the nerve from the proximally situated ganglion layer to the optic chiasma. In another instance of reversed polarity, however, the optic nerve (*n.*, fig. 28, pl. 26) passed distad through retina and tapetum and ended in the loose connective tissue surrounding the eye.

Still other variations were observed. The optic nerve of the eye shown on the right in figure 36 (pl. 28) formed along the inner (distal) surface of the ganglion layer and as a large bundle of fibers left the eye by running over the lower lip of the cup without passing through retina or tapetum. It then coursed along the ventral wall of a patent optic stalk to reach the chiasma. Unfortunately, these features are not shown in the section selected for the photomicrograph. Another eye, similar in features to the one shown in figure 23 (pl. 25), possessed an optic nerve which passed through the retina in typical fashion, then across the optic stalk, and on to the brain along the outside of the patent optic stalk. The optic nerve of yet another eye had two divisions: one from the dorsal part of the eye and one from the ventral part. The former

entered the chiasma by way of the dorsal wall of the optic stalk, the latter by way of the ventral wall of the stalk.

FACTORS DETERMINING RETINAL POLARITY

Although the evidence is limited, I think that one of the most important factors concerned in the establishment of retinal polarity is the presence in the eye of a developing lens. This conclusion is based upon observations of reversed retinal polarity in experimental eyes in which both the sensory layer of the optic cup *and* the lens placode or vesicle have been rotated. In the presence of a lens, now medially situated, the layers of the retina develop in accordance with their normal prospective fates and the fully differentiated eye appears to be looking inward. In the absence of a translocated lens, however, histological (qualitative) regulation occurs within the inverted retina and the differentiated eye appears to be looking outward. In the latter circumstance, moreover, should a lens be induced in the regenerated or engrafted ectoderm covering the rotated retina, then conditions are most favorable not only for histological but also for morphological regulation of the eye. Eyes with lenses are usually larger and better formed than eyes without lenses. For example, the two instances only of excellent regulation in series 20 were shown by animals which possessed a lens in the experimental eye.

The morphogenic role of the lens lies perhaps in its physical presence, which influences the form of the pupil, iris, chambers, and adnexa of the eye. Physical forces created by the growing and differentiating lens might set up intraretinal conditions which would orient the outgrowth of cellular processes such as nerve fibers, supporting fibers, and the outer segments of receptor cells, thereby determining the polarity of the retina. Weiss (1929) has demonstrated the importance of physical forces for the orientation of nerve fibers grown in tissue culture and has concluded (Weiss, 1933) that physical forces acting similarly upon the "ground substance" of the embryo are of considerable morphogenic significance. Perhaps also physiological (chemical) interactions between developing lens and retina have an influence upon the development of retinal polarity.

In the absence of a developing lens, in the otherwise normal eye or in the experimental eye, other morphogenic factors, normally supplementing the influence of the lens, would now be of first importance. I regard the tapetum as one of these factors, because, first, rods and cones usually differentiate at that surface of the normal or inverted retina lying nearest to a pigmented epithelium. Moreover, the more normal the physical relationship between retina and tapetum the better the differentiation of the receptor cells. This is especially well shown in a few instances (see pl. 28, fig. 39) in which a part of the retina is in contact with the tapetum, whereas the rest of the retina extends into an epithelial vesicle. The rods and cones adjacent to the pigmented epithelium are essentially normal; those not in contact with the tapetum are stunted and poorly differentiated. The outer segments of the first rods are long, straight, and thick; their cytoplasm stains heavily and evenly with eosin and their ellipsoids are large and clear. On the other hand, the outer

segments of the second rods are short, bent, and relatively thin; their cyto-plasm stains faintly and unevenly and clearly shows a striated or spiral organization; the ellipsoids are small or absent altogether. Similar differences hold also for the cones.

Other instances of a relationship between receptor cells and pigmented structures may be noted. Intraretinal lacunae lined with rods and cones frequently contain one or more masses of pigment (see pl. 26, fig. 30). In examples of vesicle formation (pl. 27, figs. 34 and 35) the receptor cells form on the distal surface of the retina, nearest to the inner, slightly pigmented epithelial lining of the cyst, whether the retina has been inverted or not. The rods and cones are poorly formed, however, under these circumstances. Indeed, the receptor cells do not develop well apart from a typical tapetum, except in the ventricles of the brain. Here differentiation is good (see pl. 27, fig. 33), although the rods and cones are not so well formed as those in the normal or well-regulated eye (see pl. 25, figs. 21–25).

How a pigmented epithelium such as the tapetum exerts a morphogenic effect on the retina is not clear. In the normal development of the eye the outer and inner layers of the optic cup are in close physical contact with each other (see pl. 23, figs. 9, 11, 13). Moreover, the definitive relationship between the ameboid, pigmented processes of the tapetal cells and the outer segments of the receptor cells is an intimate one both structurally and functionally. It is conceivable that physical and chemical factors, operating under this condition, of close association of tapetum and retina, would be of morphogenic significance. On the other hand, the role of the tapetum may not be an active one. As a heavy, perhaps impermeable membrane, the tapetum might be responsible for the establishment of gradients, a pH gradient for example, within the developing eye which might be casually related to structural polarity of the retina. Dragomirow (1936, p. 733) postulated an "instituerende Gradient" to explain the induction of retina in pieces of presumptive or early differentiated tapetum, when the latter are placed in contact with the developing lens or the otic vesicle.

DISCUSSION

DETERMINATION OF AXES OF SYMMETRY

It will be helpful, in relating this investigation to others, to review briefly the studies which have been made on the establishment of axes of symmetry in developmental systems. I hope to demonstrate that the study presented above provides an additional example of the general principle of progressive determination (see Weiss, 1939, p. 415, and Needham, 1942, p. 111), according to which an embryonic system exhibits an increasing restriction of the developmental potencies of its parts, or, conversely, a decreasing capacity for regulation.

Forelimb.—At the time when the blastopore is crescentic in shape, presumptive forelimb materials in *Triton* have not as yet become irreversibly set with regard to polarity (Rotmann, 1931). If, however, presumptive forelimb mesoderm in *Amblystoma punctatum* is removed from its position slightly

lateral to the blastopore in a large to medium-sized yolkplug stage and grafted with inverted orientation into an older embryo, a limb of reversed asymmetry will be formed (Detwiler, 1933). Only one axis, however, has become determined at this time, namely, the anteroposterior axis. The condition of a single polarized axis extends over several days of development in *Amblystoma punctatum* (Harrison, 1921), but by the late tailbud stage (Harrison's stage 35 for *Amblystoma*) the dorsoventral axis becomes irreversibly determined (Swett, 1927). Throughout the so-called tailbud stages of development in *Amblystoma* the third or mediolateral axis of the forelimb is undetermined (Harrison, 1925), but after *Amblystoma* stage 37 this axis becomes irreversibly fixed (Swett, 1927). The determination of these axes is not sudden, but gradual; *Amblystoma* stages 33 and 34 constitute a transitional period in the establishment of the dorsoventral axis, stages 35 and 36 a period of transition in the determination of the mediolateral axis (Swett, 1927, 1937).

Inner ear.—The development of the amphibian ear is strikingly similar to that of the limb (Harrison, 1936, 1945). The auditory placode of the early neurula is isotropic, that is, if the auditory placode of the medullary plate stage (*Amblystoma* stage 15) is transplanted, it develops into a normal ear with normal posture, irrespective of the orientation of the graft. By the close of neurulation (*Amblystoma* stages 20 and 21), however, the placode is polarized anteroposteriorly although it is still regulatory with respect to dorsoventral relations. Transplants from the late neurula develop normally with respect to dorsoventral relationships, regardless of their orientation, but the anteroposterior differentiation of the ear now accords with the orientation of that axis of the graft. Later in development, in the early tailbud stage (*Amblystoma* stage 25), biaxial polarization is established.

Nervous system.—There appears to be a progressive determination of anteroposterior, dorsoventral, and mediolateral axes of the central nervous system, although the evidence is not so clear as that with respect to the forelimb. The early experiments of Spemann (1912b), in which a square of medullary plate plus mesodermal substratum was rotated through 180°, showed that the anteroposterior axis of the dorsal organs was fixed at that time. Recently, Roach (1945) performed a similar rotation of the anterior part of the medullary plate in *Amblystoma punctatum* with and *without* underlying chordamesoderm. Both experimental procedures resulted in a reversed anteroposterior polarity of the rotated piece of brain. It appears, therefore, that polarity of the medullary plate exists at the beginning of neurulation, if not in preneurula stages (Roach, 1945), and that this polarity may be maintained in opposition to a reversed polarity in the mesodermal substratum. This conclusion, however, is not supported by the experiments of Alderman (1935) in which the optic anlage of *Hyla regilla* was rotated 90° and 180°. Alderman found that the rotated square of medullary plate showed regulatory development. Presumptive eye became brain, and conversely. He found also that median rostral pieces of medullary plate, underbedded with median substrate from more posterior regions of the neural plate, formed brain, not eyes. Roach has suggested that the differences between her results and those of Alderman may be

due to interspecific differences or to a difference in the size of the ectodermal square which was analyzed. Alderman worked with a small region of the medullary plate, the optic anlage, whereas Roach studied the effects of rotating all, or a lateral half of, the anterior part of the medullary plate.

The experiments of Hooker (1917) and, to a less degree, those of Wieman (1922), although performed upon postneurula stages, reinforce the conclusion that the anteroposterior axis of the nervous system is established early in development. Hooker found that if the anteroposterior axis of a segment of the neural tube of *Amblystoma punctatum* was reversed, the original structural polarity was maintained. This was indicated by the caudal growth of ascending fibers from the original anterior end of the rotated piece and the cranial growth of descending fibers from the original posterior end of the reversed segment. After a period of 18 postoperative days, however, there was, within the rotated segment—if we may judge from the normal behavior of the experimental animals—a physiological readjustment without, apparently, any structural reorganization.

On the other hand, the experiments of Detwiler involving the anteroposterior reversal of the medulla (Detwiler, 1943) and the brachial segment of the spinal cord (Detwiler, 1923) of *Amblystoma punctatum* indicate that the primary polarity of the neural tube is not determined so far as size, shape, and number of cells at any given level are concerned. Moreover, there is evidence that Mauthner's neurons, which are so highly axiate, are not irreversibly polarized in the early tailbud stage (*Amblystoma,* stage 23). In several occurrences of reversed medullae the Mauthner's neurons developed normally. The abnormal feeding behavior of the animals was attributed to the reversed condition of the auditory organs and to defective intracentral connections.

The dorsoventral axis of the central nervous system seems to be determined not later than the tailbud stage (*Amblystoma,* stage 30), according to the experiments of Hooker (1930) in which the neural tube of *Amblystoma punctatum* was rotated 90°, 135°, and 180° about its longitudinal axis. Although functional regulation was achieved by a dorsoventral decussation of the fasiculi at the cut surfaces, the rotated segment of the tube did not exhibit a regulatory reorganization. The experiments of Hutchinson (1936) give some indication that the dorsoventral axis of the neural tube of *Amblystoma punctatum* is determined even earlier, in the open medullary plate (*Amblystoma,* stage 15). By homoplastic transplantation Hutchinson' exchanged right and left lateral halves of medullary plates, thereby reversing the mediolateral (definitive dorsoventral) axis of the grafts. The transplants, instead of neurulating in conformity with the movements of the adjacent parts of the medullary plate, rolled outwardly, in accordance with their presumptive pattern of movement, to form small segments of spinal cord adjacent to the neural tube of the host. Although usually the graft later became incorporated into the central nervous system of the host and the behavior of the host was normal, Hutchinson found evidence that certain elements, especially the Rohon-Beard cells, were determined at the time of the operation. These primitive sensory cells, normally found in the dorsal regions of the neural tube,

appeared in ventral areas of the grafts, in accordance with their prospective positions. Since Rohon-Beard cells were also observed in dorsal (presumptive motor) regions of the graft, an unequivocal answer from these experiments cannot be given to the question of whether the dorsoventral axis of the neural tube is determined in the open medullary plate.

The dorsoventral axis of the neural tube is not established, according to Roach (1945), in the open medullary plate of *A. punctatum*. Exchanges between right and left lateral halves of the medullary plate, thus effecting a reversal of the mediolateral (definitive dorsoventral) axis of the grafts, resulted in regulatory development. These experiments and those of Hutchinson, although essentially alike in method and material, give different results. Unfortunately, Roach did not refer to the earlier work of Hutchinson. The experiments of Federow (reported by Braus, 1920) favor the interpretation that the mediolateral polarity of the medullary plate is fixed in the early neurula. He showed that the motor and sensory areas of the medullary plate of *Bombinator* are determined in the early neurula. Extirpation of medial or lateral parts of the medullary plate result in defects in the motor or sensory areas, respectively. Federow's experiments differ from those of Hutchinson and Roach in method and material. Simple extirpation, regarded by Harrison (1933) as a negative test of determination, and transplantation frequently do not give the same results and that which is true of a urodele may not hold for anurans.

The retina being considered, for the purposes of discussion, as a part of the brain, the experiments reported in this paper provide information on the time of determination of the definitive mediolateral axis of the central nervous system. Here, as in the forelimb, the establishment of this axis occurs relatively late in development and probably after the anteroposterior and dorsoventral axes have been determined. It has been shown that the structural polarity of the retina can be reversed as late as the early larval stage 20 (pl. 23, fig. 14) by the simple operative procedure of rotating the inner layer of the optic cup so as to reverse vitreal and tapetal surfaces. It should be emphasized that the conclusions drawn above respecting the time of determination of the mediolateral axis of the nervous system must be restricted to the conditions of the experiments reported here. Future investigation may show that *Hyla regilla* differs in this regard from other amphibians, or that the time of determination of the mediolateral axis in the central nervous system proper differs from that in the retina, or finally, that a different conclusion is reached from experiments in which the rotated piece is larger or smaller than that used by me.

Epidermis.—The problem of determination of polarity in the amphibian epidermis has not been studied sufficiently for one to make any conclusion with respect to progressive establishment of the axes of symmetry. The anteroposterior polarity of the epidermis appears in certain experiments to be fixed at the time of gastrulation or neurulation. By rotating squares of gastrular ectoderm in *Rana esculenta* and the axolotl, Woerdeman (1925) demonstrated that the direction of ciliary action is determined in the late gastrula. Twitty (1928) has shown that the direction of ciliary beat in *Amblystoma punctatum*

is fixed later, at the end of neurulation (*Amblystoma,* stages 18 and 19). Cilia developing upon a piece of ectoderm which had been rotated 180° before closure of the neural folds beat in the same direction as those on the adjacent ectoderm; cilia upon a piece of ectoderm similarly rotated *after* neurulation beat in the opposite direction. Twitty (1941) has indicated that in *Triturus torosus,* as in *A. punctatum,* polarity of the embryonic epidermis is seemingly fixed at the end of neurulation, judging from the results of a few preliminary and unpublished experiments involving the rotation of squares of lateral ectoderm at different stages of development. However, lateral ectoderm from certain postneurula embryos (*Amblystoma,* stages 26 and 29) of *T. torosus* may exhibit a "redetermination" of the original pattern of ciliary currents when it is transplanted to the region of the developing dorsal fin in embryos of *T. rivularis* or *T. torosus;* in these experiments the transplant contributed to the formation of a segment of the fin, and the pattern of ciliary action on the graft became adjusted to that of the new environment by a series of orderly shifts. Twitty concluded that the regulation observed here was owing to the strong morphogenic environment in which the graft had been placed, whereas the absence of regulation in earlier experiments (Woederman, 1925; Twitty, 1928) was due to the fact that the rotated piece of prospective epidermis was situated in a weak environment which was unable to modify the ciliary polarity already established. Twitty found also that epidermis from still older embryos (*Amblystoma,* stage 31) would not regulate even if placed in the region of the developing fin, although it is conceivable, as he suggests, that the ciliary polarity of epidermis from even stage 31 might be reversed by a still stronger environment.

It might be pointed out that the fixation of ciliary polarity with which Twitty is dealing may be largely a physiological problem and not entirely comparable to a problem of structural polarity such as that presented in this paper. It will be recalled that Hooker (1917) obtained a functional regulation in segments of the spinal cord which had been rotated end for end although the original structural polarity was apparently retained. It seems more probable, however, that either a change in structure, perhaps at a molecular level, precedes a reversal of some physiological process or that a change in physiological polarity is followed by a change in structural organization. In other words, it is unlikely that physiological and structural polarity can be dissociated.

Luther (1934) found that the mediolateral axis of the presumptive epidermis in *Triton* was determined at the close of neurulation. Pieces of ectoderm the inner and outer surfaces of which were reversed before this time regulated into normal, ciliated epidermis. Pieces inverted after neurulation did not heal, owing to the action of cilia which had already differentiated. Of the studies on polarity mentioned above, these experiments are most similar to those reported in this paper. Yet even Luther's experiments are not wholly comparable. First, Luther reversed the inner and outer surfaces of a two-layered, cellular epithelium; I rotated a much thicker, probably syncytial, layer of epithelium. Second, Luther was not able to maintain pieces of epider-

mis in the inverted position after they had differentiated cilia, whereas in my experiments there were no insurmountable difficulties to maintaining an inverted piece of early differentiated retina. It is possible, although unlikely, that had Luther's pieces of ciliated ectoderm healed in an inverted position, the cilia might have been resorbed and others differentiated on the new external surface. Barring this possibility, it seems that the mediolateral polarity of the amphibian epidermis is determined much earlier in development than that of the neural tube (retina). This is probably owing to the fact that organelles such as cilia are precociously differentiated in the epidermis.

Entoderm.—The anteroposterior polarity of the entoderm is determined at least by the middle of neurulation as shown by Kemp (1946). Several operative procedures, including an end-for-end reversal of the lateral half of the neurula of *Hyla regilla,* reveal a fixity of polarity in the archenteron at the time of operation. Presumptive buccal and cloacal epithelia, presumptive oesophagus and colon, presumptive stomach and intestine may develop side by side to form a digestive tube but without any histological regulation.

The experiments of Holtfreter (1933) on exogastrulation in the axolotl show that mediolateral regulation of the entoderm is possible, provided the differentiating entodermal epithelium is brought into a particular relationship with developing connective tissue and a free surface. In some exogastrulae, entodermal cells which had not invaginated, owing to the failure of the gastrulation, formed a mucosal lining about an internal cavity. The inner surfaces of the cells adjacent to the lumen were clearly secretory in function, whereas the outer surfaces next to the mesenchyme were clearly basal in structure. Normally, as a result of gastrulation, the outer or distal surfaces of the entodermal cells become the secretory ends of the cells, the inner surfaces the basal ends of the mucosal cells.

Conclusion.—In the developmental systems discussed above there appears to be a progressive determination of the axes of symmetry in the following order: first, anteroposterior axis; second, dorsoventral axis; third, mediolateral axis. Table 4 summarizes the probable times of determination of these axes of symmetry in those developmental systems of the amphibian ambryos which are considered here. We still lack information on the establishment of the mediolateral axis of the inner ear and of the dorsoventral axis in the epidermis and entoderm, if one exists at all in these structures. We need, moreover, a clearer understanding of the time of determination of the dorsoventral axis of the neural tube. The studies on the development of retinal polarity in *Hyla regilla* presented in this paper provide information on the time of determination of the mediolateral axis of the neural tube.

REGULATORY DEVELOPMENT OF THE EYE

An account of the developmental history of the cells which give rise to the amphibian eye will further illustrate the general principle of progressive determination with diminishing regulation. Needham (1942, p. 111) has compared the progressive restriction of developmental potencies to a series of cones. "At the top of the uppermost cone there is a ball in a position of ex-

tremely unstable equilibrium. It will tend to fall along the side of the cone and will reach a point at some one of the 360 degrees of the cone's circumference. Here it will again find itself in a position of unstable equilibrium, only now with respect to a second stage of determination and will again be pushed in one direction or another, again to occupy a passing equilibrium, and so on until the final stage of absolute stability is reached, i.e., the plan of the adult body." Applying this analogy to the development of the rods and cones of the eye, we have the picture shown in figure *B*.

TABLE 4

Time of Determination of Axes of Symmetry

Developmental systems	Anteroposterior axis	Dorsoventral axis	Mediolateral axis
Forelimb.......	Mid-gastrula *Amblystoma* Detwiler (1933)	Late tailbud embryo *Amblystoma* Swett (1927)	Early larva *Amblystoma* Swett (1927)
Ear............	Late neurula *Amblystoma* Harrison (1936)	Early tailbud embryo *Amblystoma* Harrison (1936)	....
Neural tube.....	Early neurula *Triton* Spemann (1912*b*) *Amblystoma* Roach (1945)	Late neurula or early tailbud embryo (?) (see discussion)	Early larva *Hyla* (retina) Eakin (see conclusion herein)
Epidermis......	Late neurula *Amblystoma* Twitty (1928)	....	Completed neurula *Triton* Luther (1934)
Entoderm.......	Early neurula *Hyla* Kemp (1946)	....	Undetermined in exogastrulae Axolotl Holtfreter (1933)

Mesectoderm.—The topmost cone (1) represents mesectoderm and the ball at its apex a mesectodermal cell. The ball may roll down this cone in any direction and onto cones of a second order situated about the base of cone 1. While the ball is rolling down the first cone its course is easily altered. For example, a ball rolling toward cone 4 may be deflected toward cone 3 or even toward cone 2 as indicated by the arrows. By analogy, a mesectodermal cell exhibits a high degree of regulation in an early period of development. Its fate—epidermis, medullary plate, chorda, somites, lateral plate, etc.—is the function of its position. This has been demonstrated by the normal development resulting from reciprocal exchanges of presumptive epidermis and medullary plate (Spemann, 1921) or exchanges of presumptive epidermis and presumptive lateral plate (Mangold, 1923). It has also been shown that presumptive skin can become chorda and function as an organizer (Spemann and Geinitz, 1927) or that even presumptive chorda may form epidermis or medullary plate (Töndury, 1936; Eakin, 1939).

Once the ball has rolled onto one of the cones at the second level, such as cone 3, there is little or no chance of transferring it to other cones in that level,

such as cones 2 and 4. This means that once the broad fate of a mesectodermal cell has been determined—skin, nervous tissue, notochord, etc.,—its developmental potencies have become restricted. Thus, definitive medullary plate transplanted into skin ectoderm will form nervous tissue and not epidermis; it no longer develops neighborwise, but now selfwise.
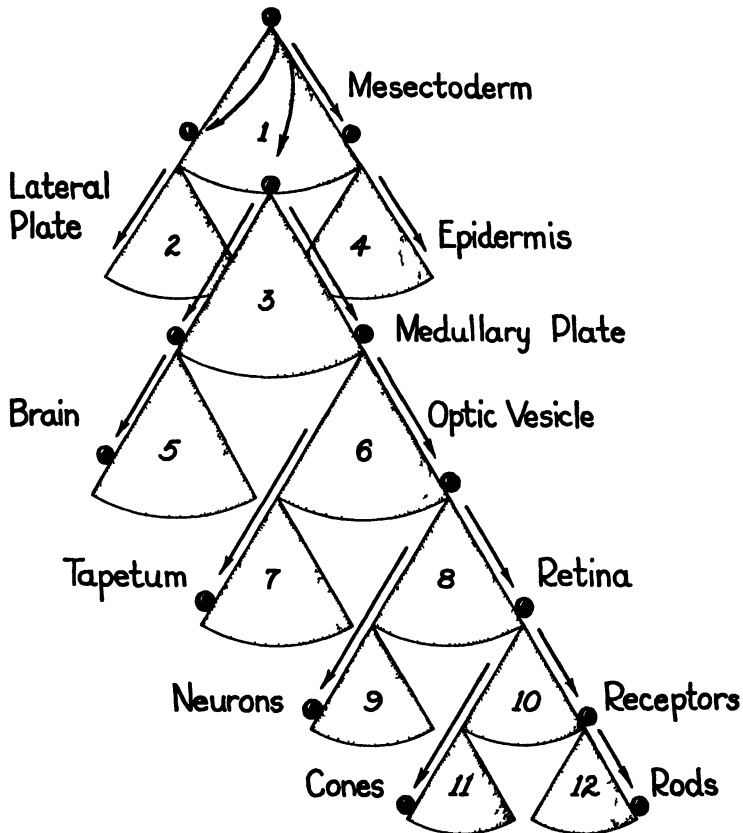

Figure B

Medullary plate.—Assuming that the ball which rolled down cone 1 (mesectoderm) connected with the apex of cone 3 (medullary plate), it would proceed in any direction to the base of this cone and to the third order of cones such as 5 (brain) and 6 (optic vesicle). Other cones not shown here might represent spinal cord, infundibulum, etc. Again the path of the ball down cone 3 is subject to change. This implies that, for a time, regulation *within* the medullary plate is possible; and indeed, as stated above (p. 261), there is experimental evidence that this is true. Alderman (1935) has shown that regions in the medullary plate the prospective significance of which is eye may become brain, and conversely. Roach (1945) obtained normal development following the exchange of right and left lateral halves of the anterior part of the medullary plate, indicating that regions of the neural plate des-

tined to form dorsal parts of the nervous system may be made to develop ventral structures, and conversely.

Optic vesicle.—Extending the simile of the system of cones, the optic vesicle (cone 6) gives rise to tapetum (cone 7) and retina (cone 8). During the time the ball is rolling down cone 6 its path is regulatory, meaning that the optic vesicle is essentially a harmonious equipotential system. A half of the whole can still form a whole, although of small size (see, e.g., figs. 36 and 37, pl. 28). On the other hand, two optic anlagen may develop into a single, large, but normally formed cyclopian eye as the result of certain operative procedures (Mangold, 1931) or owing to a decrease in oxygen tension (Detwiler and Copenhaver, 1941), to mention only two examples from the literature on cyclopia. More striking instances of regulation, both qualitative and quantitative, are provided by the experiments of Detwiler (1929) and Pasquini (1927), in which two optic vesicles of *Rana fusca* and *Pleurodeles waltli*, respectively, were fused. The result was an essentially normal eye. Undoubtedly, cells, the prospective fate of which was tapetum became retina, and conversely. More exact evidence that this interchange is possible is given by the studies of Dragomirow (1932, 1934, 1936). She obtained regulatory development of a piece of tapetum from the optic cup of several types of amphibians, including both urodeles and anurans. A small segment of the outer (tapetal) layer of the optic cup, when transplanted between lens and cornea, differentiated into a small optic cup consisting of both tapetum and retina. The same result was obtained if the graft was placed next to the auditory vesicle. In these experiments some of the tapetal cells which had already become visibly differentiated from the inner layer of the optic cup, regulated to form neural and sensory elements instead of pigmented epithelium. Regulation was obtained in the tapetum of *Triton taeniatus* as late as Harrison's stage 32. Dragomirow obtained instances of the opposite type of regulation, namely, the development of tapetum, as well as retina, from pieces of the inner layer of the optic cup. Prior to Harrison's stage 24 the presumptive retina in *T. taeniatus* is regulative; by stage 29, however, the inner layer of the optic cup, especially the central part, is determined for retina alone.

Retina.—The experiments reported in this paper show that regulation *within* the retina is possible long after the optic cup has fully formed, indeed, until the retina begins to differentiate histologically into sensory and neural elements (*Hyla,* stage 22). A ball proceeding from cone 6 (optic vesicle) to cone 8 (retina) may roll in the direction of cone 9 (neurons) or cone 10 (receptors), and, as is indicated by my experiments, the path is modifiable until very late in the development of the eye.

Rods and cones.—Extrapolating, so to speak, the simile of the cones to the differentiation of the receptor cells, one may assume that within the outer layer of the retina regulatory development may be possible even after the several layers have been determined. For this there is as yet no experimental evidence. It is not unlikely, however, that just prior to cellular differentiation a potential cone could become a rod, and conversely.

SUMMARY

1. The presumptive or early differentiated retina of the Pacific tree frog, *Hyla regilla,* was rotated so as to reverse the inner (vitreal) and outer (tapetal) surfaces in embryos and larvae ranging from the early tailbud embryo (stage 16) to free-swimming larvae (stages 20–22). A total of 218 successful operations were performed; 179 experimental animals were sectioned and examined histologically.

2. The experimental animals were classified according to the following types of structural organization in the inverted retina: (1) good regulation, i.e., both histological (qualitative) and morphological (quantitative); (2) fair regulation, i.e., histological only; (3) reversed polarity; and (4) disorganization (see table 1, p. 247).

3. High proportions of regulation were observed in experimental eyes the retina of which had been rotated in stages 16 to 20. Operations on larvae older than stage 20 resulted in no examples of good regulation and only a few, some doubtful, of fair regulation.

4. Only 5 instances of reversed polarity were found among experimental animals from series (stages) 16 to 20. Of these, one was doubtful and three were instances in which the lens vesicle had been rotated together with the retina. After stage 21, however, the proportion of experimental eyes exhibiting reversed polarity was high.

5. It is concluded that stage 21 in *Hyla regilla* is a critical time in the development of the retina. Before that stage, retinal polarity is not irreversibly determined; presumptive sensory and neural elements are interchangeable. Thereafter, retinal polarity is irreversibly determined and there is no evidence of further regulation along the polar axis of the retina when tested by the experimental procedure of rotating the developing retina so as to reverse its inner and outer surfaces.

6. The lens vesicle and the tapetum are suggested as probable morphogenic factors in the establishment of retinal polarity. Physical forces created by the growing and differentiating lens are believed to orient the outgrowth of cellular processes as suggested by Weiss for nerve fibers. The tapetum might be involved in the establishment of physiological gradients causally related to the determination of retinal polarity.

7. A series of stages in the normal development of *Hyla regilla* is described, figured, and numbered to agree with stages already described for other anurans.

8. Microscissors of simple construction, which were developed for this study, should be useful in many investigations involving microsurgery.

9. A review of the experimental studies on the determination of the axes of symmetry is presented in the discussion of the results of this investigation.

10. A graphic representation of the concept of progressive determination, originally figured by Needham, is here developed and used to illustrate progressive determination in the developing amphibian eye.

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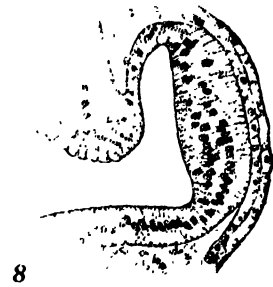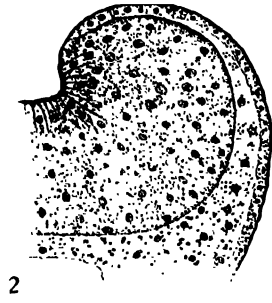
PLATES

PLATE 22

Stages 15–18 in normal development of *Hyla regilla*; external features and appearance of developing eye as seen in transverse sections.

Figs. 1 and 2. Stage 15. Length, 1¾ mm.; neural folds high and unfused in cranial part of neurula but in contact with each other in and posterior to region of hindbrain; presumptive optic vesicle indicated by intensely pigmented depression in ventrolateral wall of future forebrain.

Figs. 3 and 4. Stage 16. Length, 2 mm.; neural tube completely closed; sense and gill plates visible; optic vesicle forming as shallow, thick-walled diverticulum of forebrain.

Figs. 5 and 6. Stage 17. Length, 2½ mm.; tailbud separated from body proper by ventral notch; stomodeal depression slight; nonmotile; optic vesicle finger-like outpocketing.

Figs. 7 and 8. Stage 18. Length, 3 mm.; tail about ⅓ length of body; caudal fin appearing; suckers indicated by two heavily pigmented areas joined medially by narrow pigmented band below stomodeum; beginning of muscular response to touch (simple flexure); optic vesicle fully formed and consisting of thick distal wall, relatively thin sides, and narrowing stalk.

PLATE 23

Stages 19–21 in normal development of *Hyla regilla*; external features and appearance of developing eye as seen in transverse sections.

Figs. 9 and 10. Stage 19. Length, 4⅓ mm.; tail rounded, more than ⅓ length of body, nasal placodes indicated by pigmented shallow depressions; suckers no longer joined by pigmented band; coil response to touch; beginning of heart beat; optic vesicle invaginating to form optic cup; lens placode invaginating into optic cup.

Figs. 11 and 12. Stage 20. Length 5⅓ mm.; tail less rounded, almost ½ length of body; appearance of external gills, with circulation; melanophores appearing on dorsal part of body; brief and weak swimming movements; optic cup fully formed and consisting of thick, inner sensory layer and thin, outer tapetal layer, faintly pigmented at back of eye; lens vesicle formed but undifferentiated; optic stalk disappearing.

Figs. 13 and 14. Stage 21. Length, 6 mm.; tail pointed, ½ length of body; external gills branching; nasal pits deep; sustained and strong swimming movements; lens vesicle faintly visible through cornea; proximal wall of lens increasing in thickness and encroaching upon cavity of vesicle; optic nerve beginning to form.

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PLATE 21

Stages 22-24 in normal development of *Hyla regilla*; external features and appearance of developing eye as seen in transverse sections.

Figs. 15 and 16. Stage 22. Length, 6½ mm.; tail longer than body; pigmentation on dorsal aspect of eye visible externally; retina differentiated into relatively narrow inner layer of nuclei (ganglion cells) and wide outer layer of nuclei separated by narrow band of white matter (inner molecular layer); rods and cones beginning to differentiate outer parts; lens fibers forming; optic nerve well developed.

Figs. 17 and 18. Stage 23. Length, 7 mm.; proportion of tail to body about 4 to 3; operculum beginning to form; colon differentiated and bent dorsally; eye completely pigmented; retina further differentiated by formation of outer molecular layer separating nuclei of rods and cones (outer nuclear layer) from nuclei of bipolar neurones (inner nuclear layer); outer parts of rods and cones better differentiated, especially ellipsoid.

Figs. 19 and 20. Stage 24. Length, 7¼ mm.; operculum covering gills; gut S-shaped; spacious pleuroperitoneal cavity ventral to colon; dorsal fin bowed; cavity of lens vesicle obliterated; outer segment, ellipsoid, and perhaps myoid of rods and cones well formed.
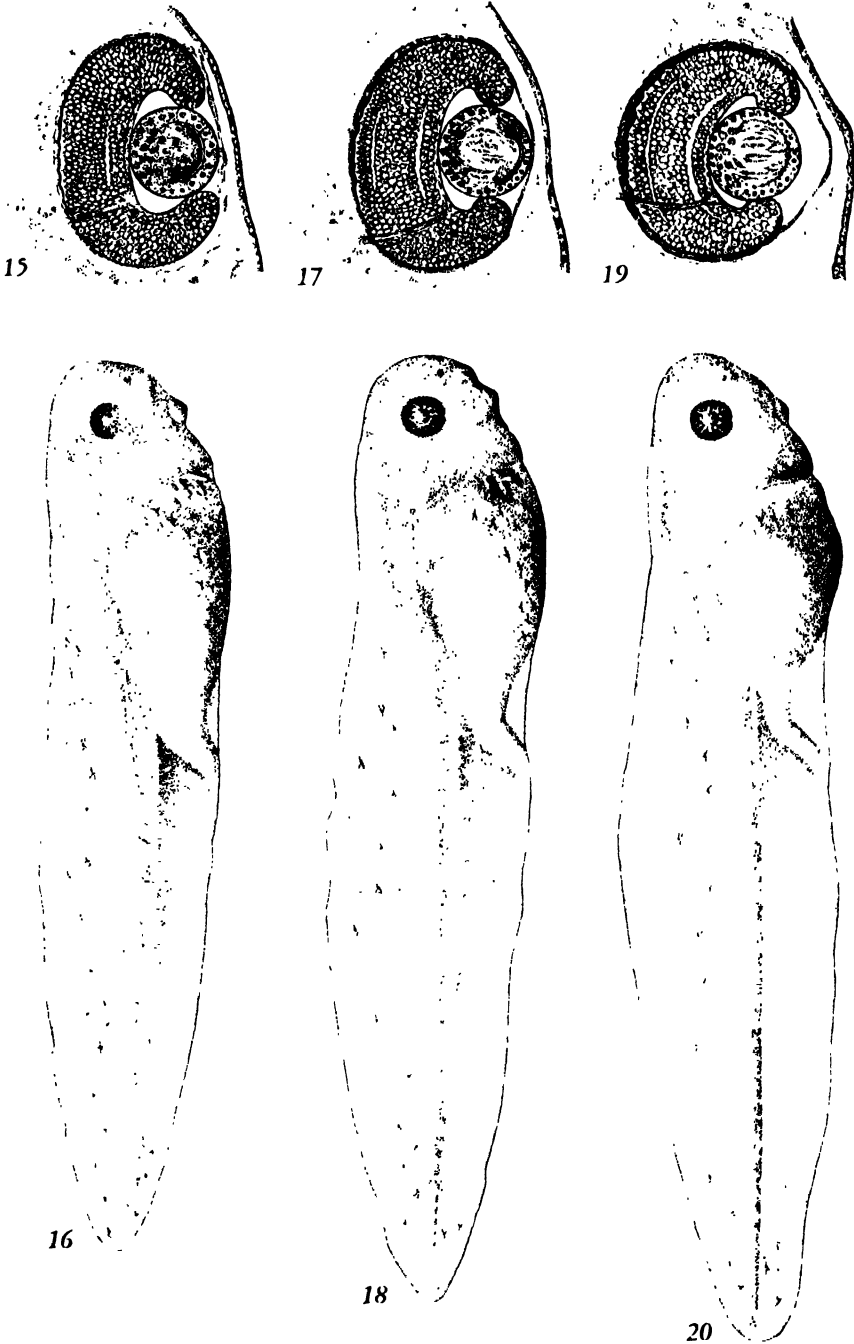
15   17   19

16   18   20

Examples of "good regulation" in experimental animals of *Hyla regilla* in which presumptive retina of right eye was rotated so as to reverse inner (vitreal) and outer (tapetal) surfaces. Note normal form of eyes (morphological regulation) and normal polarity and pattern of retinae (histological regulation).

Fig. 21. Excellent regulation in experimental eye of *Hyla* tadpole, operated in stage 16. *g.*, ganglion layer; *i.m.*, inner molecular layer; *i.n.*, inner nuclear layer; *o.m.*, outer molecular layer; *o.n.*, outer nuclear layer; *r.*, layer of rods and cones. × 135.

Fig. 22. Excellent regulation in experimental eye of *Hyla* tadpole, operated in stage 17. × 135.

Fig. 23. Good regulation in experimental eye of *Hyla* tadpole, operated in stage 16. Note: neural tissue (*nt.*) below eye and dorsally between eye and brain; patent optic stalk (*op.*); degenerating ganglion cells (*g.*). × 115.

Fig. 24. Good regulation in experimental eye of *Hyla* tadpole, operated in stage 18. Intraretinal lacuna owing to infolding of receptor layer. *n.*, optic nerve. × 135.

Fig. 25. Excellent regulation in experimental eye of *Hyla* tadpole operated in stage 19. *e.*, ellipsoid; *o.*, outer segment of receptor cell (rod). × 150.
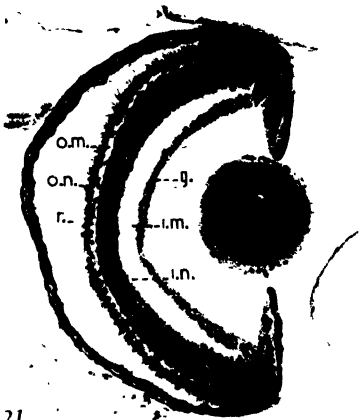
(Unretouched photomicrographs)

Experimental eyes in tadpoles of *Hyla regilla* in which presumptive or early differentiated retina was rotated so as to reverse inner (vitreal) and outer (tapetal) surfaces.

Fig. 26. "Good regulation" in experimental eye of *Hyla* tadpole, operated in stage 20. Morphology of eye is fair; retina, aside from irregularities, is normal in polarity and pattern. × 180.

Fig. 27. Example of "fair regulation" in experimental eye of *Hyla* tadpole, operated in stage 20. Ocular morphology is poor; retina, however, exhibits normal polarity and arrangement of sensory and neural elements (histological regulation). × 160.

Fig. 28. Example of "reversed polarity" in experimental eye of *Hyla* tadpole, operated in stage 22. Note: distal position of receptor cells (r.); proximal position of ganglion cells (g.); optic nerve (n.) passing distad toward skin; large blood vessel or sinus (s.); epithelial vesicle (ep.). Tapetum of host origin. × 150.

Fig. 29. Experimental eye of *Hyla* tadpole, in which sensory layer of optic cup *plus* lens vesicle was rotated in stage 19. Note: proximal position of lens; inner half of retina normally polarized with respect to translocated lens; outer half of retina normally polarized with respect to transverse axis of body (histological regulation). × 140.

Fig. 30. Example of "disorganization" in experimental eye of *Hyla* tadpole in which presumptive retina was reversed in stage 18. Receptor cells may be seen dorsally, ventrally, and lining a large intraretinal cavity; nuclear and molecular layers are not arranged according to a regular pattern. × 150.
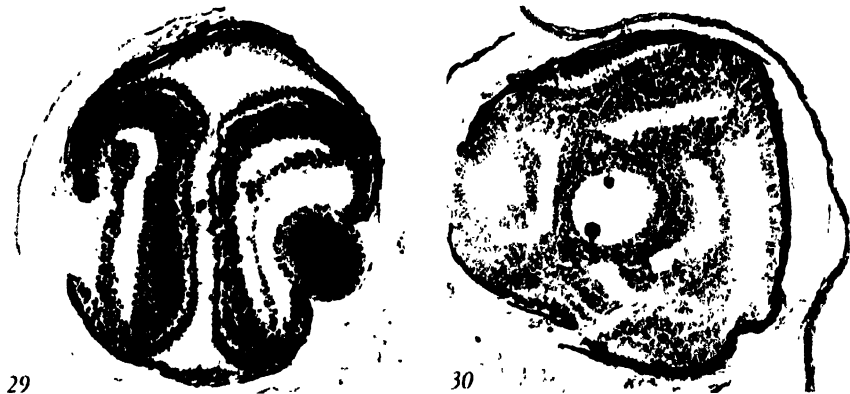
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Fig. 31. Example of "reversed polarity" in *Hyla* tadpole in which sensory layer of optic cup was rotated in stage 22. Note distal position of rods and cones and proximal position of ganglion cells. Tapetum of donor origin. *ep.*, epithelial vesicle. × 125.

Fig. 32. Example of "reversed polarity" in *Hyla* tadpole in which sensory layer of optic cup was rotated in stage 22. Retina, although extensively folded, shows reversed orientation of sensory and neural elements. Tapetum of donor and host origin. *r.*, receptor cells. × 155.

Fig. 33. Instance of accidental transplantation of presumptive retina of *Hyla* embryo in stage 16 to a ventricle of the brain. Note distal position and good differentiation of rods and cones. × 115.

Fig. 34. Example of an epithelial vesicle formed distal to retina in *Hyla* tadpole in which sensory layer of optic cup was rotated in stage 20. Note: horseshoe shaped retina with rods and cones distally situated; mass of tapetal cells at base of retina; thin pigmented epithelium lining vesicle (*ep.*). Compare with fig. 35. × 115.

Fig. 35. Example of an epithelial vesicle formed distal to retina in *Hyla* tadpole from which cornea and lens were removed in stage 20 but retina was not inverted (control). Note great similarity of features shown here with those in fig. 34. *ep.*, epithelial vesicle. × 115.
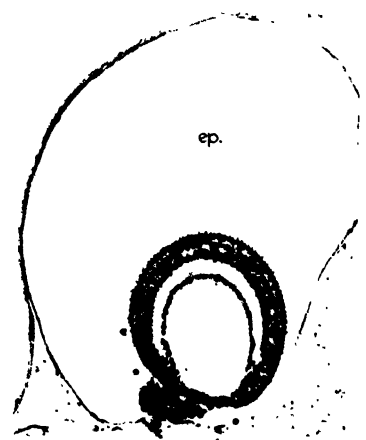
PLATE 28

(Unretouched photomicrographs)

Fig. 36. A double eye in *Hyla* tadpole in which retina, rotated in stage 16, became divided and regulated into two optic cups, each surrounded by tapetum. Eye on right possessed an optic nerve; one on left lacked an optic nerve. Note degenerating ganglion cells (*g.*) in the latter. × 150.

Fig. 37. Experimental eye in *Hyla* tadpole in which retina *plus* lens vesicle was rotated in stage 18. Dorsal and ventral halves of retina are organized with reference to lens, giving appearance of a double eye. Compare with fig. 29. × 140.

Fig. 38. Eye of control larva of *Hyla* fixed 24 hours after cornea and lens vesicle had been removed in stage 22 but retina left undisturbed. Note: blood filled vesicle (*cp.*) developing between retina and skin, which had been engrafted over wound; early curling of dorsal margin of retina distad into vesicle; recession of dorsal part of tapetum; anchoring effect of optic nerve. *bc.*, blood cells; *m.*, mesoderm transplanted with skin ectoderm. × 160.

Fig. 39. Eye of control tadpole of *Hyla* in which cornea and lens vesicle had been removed in stage 20 but retina left undisturbed. Note: large fluid-filled epithelial vesicle (*cp.*); curling of dorsal part of retina; recession of dorsal part of tapetum; stunted and poorly differentiated rods and cones extending into vesicle; excellent differentiation of receptor cells adjacent to tapetum. × 185.

Fig. 40. "Reversed polarity" in experimental eye of *Hyla* tadpole in which sensory layer of optic cup was rotated in stage 20. Note: rods and cones at distal surface of retina (toward lower border of photomicrograph); optic nerve (*n.*) passing mesiad directly from ganglion layer to brain; epithelial vesicle (*cp.*) distal to eye. × 165.

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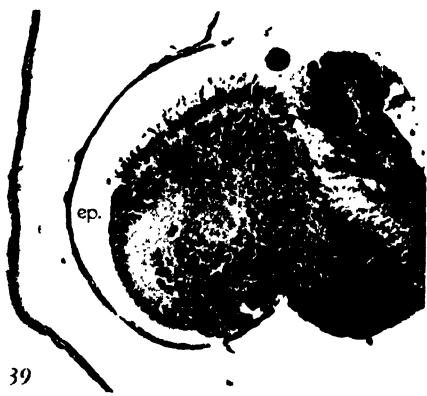
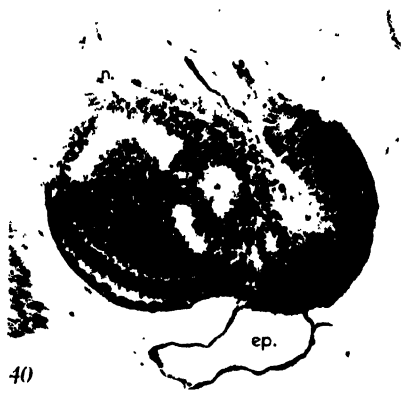38

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